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# Conformational instability of the N- and C-terminal lobes of porcine pepsin in neutral and alkaline solutions

XINLI LIN,[1] JEFFREY A. LOY,[1] FREDY SUSSMAN,[1] AND JORDAN TANG[1,2]

[1] Protein Studies Program, Oklahoma Medical Research Foundation, and [2] Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

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**Abstract**

Pepsin contains, in a single chain, two conformationally homologous lobes that are thought to have been evolutionarily derived by gene duplication and fusion. We have demonstrated that the individual recombinant lobes are capable of independent folding and reconstitution into a two-chain pepsin or a two-chain pepsinogen (Lin, X., et al., 1992, *J. Biol. Chem. 267*, 17257–17263). Pepsin spontaneously inactivates in neutral or alkaline solutions. We have shown in this study that the enzymic activity of the alkaline-inactivated pepsin was regenerated by the addition of the recombinant N-terminal lobe but not by the C-terminal lobe. These results indicate that alkaline inactivation of pepsin is due to a selective denaturation of its N-terminal lobe. A complex between recombinant N-terminal lobe of pepsinogen and alkaline-denatured pepsin has been isolated. This complex is structurally similar to a two-chain pepsinogen, but it contains an extension of a denatured pepsin N-terminal lobe. Acidification of the complex is accompanied by a cleavage in the *pro* region and proteolysis of the denatured N-terminal lobe. The structural components that are responsible for the alkaline instability of the N-terminal lobe are likely to be carboxyl groups with abnormally high p$K_a$ values. The electrostatic potentials of 23 net carboxyl groups in the N-terminal domain (as compared to 19 in the C-terminal domain) of pepsin were calculated based on the energetics of interacting charges in the tertiary structure of the domain. The groups most probably causing the alkaline denaturation are $Asp^{11}$, $Asp^{159}$, $Glu^{4}$, $Glu^{13}$, and $Asp^{118}$. Especially, the partially buried $Asp^{11}$, which interacts with $Asp^{159}$, could cause one of these two groups to have an abnormally high p$K_a$ and the other an abnormally low p$K_a$ value. Thus, the ionization of $Asp^{11}$ at a high pH may place two negatively charged residues in close vicinity. This unfavorable situation may be the trigger for the denaturation of the N-terminal lobe of pepsin.

**Keywords:** carboxyl ionization; denaturation; domain structure; electrostatic potentials; porcine pepsin

The fact that some aspartic proteases, such as HIV protease and renin, are involved in human diseases has stimulated much interest recently in the understanding of the structure–function relationships of this group of enzymes.

Pepsin is a particularly good model for the study of aspartic proteases because detailed information is available on its structure (Abad-Zapatero et al., 1990; Cooper et al., 1990; Sielecki et al., 1990), enzymic properties (Fruton, 1976), and zymogen activation (Tang & Wong, 1987; Hartsuck et al., 1992). Like other eukaryotic aspartic proteases, pepsin is a single-chain protein, and its tertiary structure consists of two homologous lobes. The substrate binding cleft and the catalytic aspartic residues are located between these lobes (for review, see Davies [1990]). On the other hand, the retroviral aspartic proteases, including the HIV protease, are homodimers with the substrate binding cleft and the active-site aspartic residues equally contributed by the two monomers (Foundling et al., 1989; Lapatto et al., 1989; Miller et al., 1989; Wlodawer et al.,

Reprint requests to: Jordan Tang, Protein Studies Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, Oklahoma 73104.

*Abbreviations:* bis-Tris, bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane; FPLC, fast protein liquid chromatography (chromatograph); HIV, human immunodeficiency virus; PAGE, polyacrylamide gel electrophoresis; *pep*, the $NH_2$-terminal lobe of pepsin; *pep·sin*, two-chain pepsin; $pepsin_a$, alkaline-inactivated pepsin; *pro*, propeptide; *propep*, the $NH_2$-terminal lobe of pepsinogen; *propep*·$pepsin_a$, complex between *propep* and alkaline-inactivated pepsin; *propep·sin*, two-chain pepsinogen; SDS, sodium dodecyl sulfate; *sin*, the C-terminal lobe of pepsinogen or pepsin; Tris, tris(hydroxymethyl)aminomethane.

1989; Fitzgerald et al., 1990). It has been proposed that the internally homologous lobes of pepsin and other eukaryotic aspartic proteases are evolutionarily derived by gene duplication and fusion, and that a primordial aspartic protease would have a homodimeric structure (Tang et al., 1978). The close relationships of pepsin and retroviral aspartic protease active-site structures and their overall folding patterns (Rao et al., 1991) suggest that the retroviral protease is closely related to a primordial form of aspartic protease. It is therefore interesting to compare the individual domains of the eukaryotic aspartic protease with the monomers of the retroviral aspartic protease (Kinemage 1).

We have reported recently that the individually expressed recombinant N-terminal lobe of pepsinogen, called *propep*, and the C-terminal lobe, called *sin*, are capable of individual folding and reconstitution into active two-chain pepsinogen, *propep·sin* (X. Lin et al., 1992). Upon activation in acid, *propep·sin* can yield two-chain pepsin, *pep·sin*. Additionally, either *propep* or *pep* can self-associate to form proteolytically active homodimer. These results suggest that the N- and C-terminal lobes of pepsin have retained some of the features of the homodimeric enzymes, especially in the ability of individual lobes to fold independently and in interlobal recognition.

The ability for folded domains of *pep* and *sin* to recognize each other and to generate activity provides an opportunity to distinguish the conformationally folded lobes from the unfolded lobes in pepsin. It has long been known that pepsin is spontaneously inactivated in neutral and alkaline solutions. By adding the individually folded recombinant lobes in an activity-reconstituting assay, we have now demonstrated that the alkaline inactivation of pepsin is caused by the denaturation of the N-terminal lobe, *pep*. Structural components possibly responsible for the alkaline denaturation of the *pep* domain have also been analyzed and identified by energy calculations.

## Results

### *Alkaline sensitivity of the two lobes of pepsin*

An experiment was designed to determine the relative sensitivity of two lobes in pepsin to inactivation at different pH values. The results in Figure 1 demonstrated that pepsin was completely inactivated at pH 8 (left column). When prefolded recombinant *sin* was added to the inactivated pepsin, no activity was generated (right column). The addition of prefolded recombinant *propep* resulted in a dramatic restoration of activity (center column). The intrinsic enzyme activity of *propep* (X. Lin et al., 1992) is not detectable in a control under the experimental conditions (results not shown). So the regenerated activity must have come from the recombination of *propep* with alkaline-inactivated pepsin. These results suggest that alkaline solutions specifically denature the N-terminal lobe (*pep*) of pepsin but retain the conformation of the C-terminal lobe, *sin*.

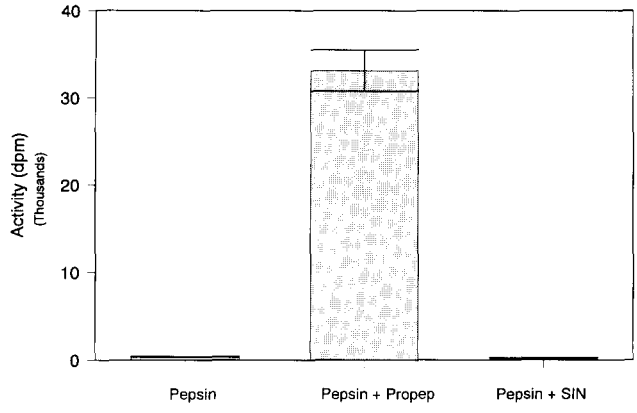


**Fig. 1.** Regeneration of activity of alkaline-inactivated pepsin by recombinant N-terminal lobe, *propep*. Pepsin (0.1 mg/mL) was incubated in 20 mM Tris-HCl (pH 8.0) at 4 °C for 20 h, and then 1 μL was taken for enzyme assay using [$^{14}$C]hemoglobin as substrate (left column). In the center column, 39 μL of partially purified recombinant *propep* (by S-300 Sepharose gel column; X. Lin et al., 1992) was incubated with 1 μL of the denatured pepsin at 20 °C for 20 min and then assayed for proteolytic activity. In the right column, denatured pepsin was preincubated with purified recombinant *sin* prior to the enzyme assay. Under the experimental conditions, recombinant *propep* showed virtually no activity. The bars at the top of the center column represent the standard deviation from three determinations.

### *Purification of propep·pepsin$_a$ complex*

The restoration of the activity of alkaline-inactivated pepsin, pepsin$_a$, by recombinant fragment *propep* must have resulted from the binding of the conformationally intact *sin* in pepsin$_a$ to *propep* to form a complex, *propep*·pepsin$_a$. For further studies, this complex was prepared in a larger scale by diluting a urea solution of recombinant *propep* into a solution of alkaline-inactivated pepsin (see Materials and methods). This procedure took advantage of the presence of pepsin$_a$ during the refolding of *propep* and thus gave a higher yield of the complex than from the mixing of components refolded separately. The lower yield in the procedure where refolding took place separately was probably caused by the self-association of *propep* after refolding. *Propep*·pepsin$_a$ complex was purified by chromatography on Sephacryl S-300 (not shown) and QAE columns. The latter gave rise to a single protein peak (Fig. 2). Neither the refolded *propep* nor pepsin$_a$ forms a peak on QAE-column chromatography. The purity of the complex was also analyzed by FPLC on a Superose 12 column, which resulted in a single peak with a shoulder on the lower molecular weight side (Fig. 3A). The shoulder probably does not represent chemical heterogeneity and is likely to be due to the lack of conformational uniformity in the denatured *propep* domain. Some heterogeneity in the conformation of refolded

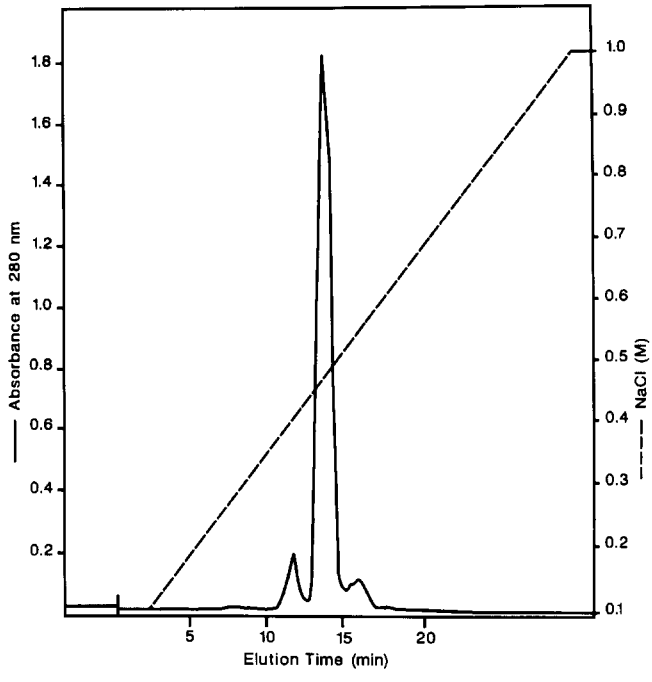


**Fig. 2.** FPLC fractionation of *propep*·pepsin$_a$ on a MonoQ column. Active fractions from Sephacryl S-300 chromatography (not shown) were applied to a MonoQ column in a Pharmacia FPLC, which had been equilibrated with 20 mM bis-Tris, 0.8 M urea, pH 6.0. Elution was effected with a linear gradient of 1 M NaCl in the same buffer.

recombinant *propep* (X. Lin et al., 1992) may also contribute to this chromatographic behavior. The conformational but not the chemical origin of the shoulder is supported by the results from SDS-PAGE (Fig. 4, lane 3), which revealed that the purified complex consists of a pepsin band (its mobility usually corresponds to near 40 kDa) and a *propep* band (near 25 kDa).

### *Acid-activation of propep·pepsin$_a$ complex*

Like pepsinogen, the complex *propep*·pepsin$_a$ is capable of activation in acid with a concomitant cleavage in the *pro* peptide region. Acid-treated complex eluted from a molecular weight-calibrated gel-filtration column (Y. Lin et al., 1992) at an eluent volume corresponding to a smaller size (Fig. 3B) than that of the untreated complex (Fig. 3A). The apparent size of the purified complex is between 150 and approx. 200 kDa (Fig. 3A). (The high apparent size is caused by the presence of a denatured N-terminal lobe in pepsin$_a$.) After acidification, the apparent size was reduced to 53 kDa (Fig. 3B). On SDS-PAGE, the *propep* band in the acid-treated (15 min at pH 2) complex is smaller (22 kDa; Fig. 4, lane 4) than from the untreated sample (25 kDa; Fig. 4, lane 3). The 22-kDa band represents an intermediate in acid activation of *propep* in the *propep*·pepsin$_a$ complex, since it is larger than the *pep* band (20 kDa; Fig. 4, lane 5) produced

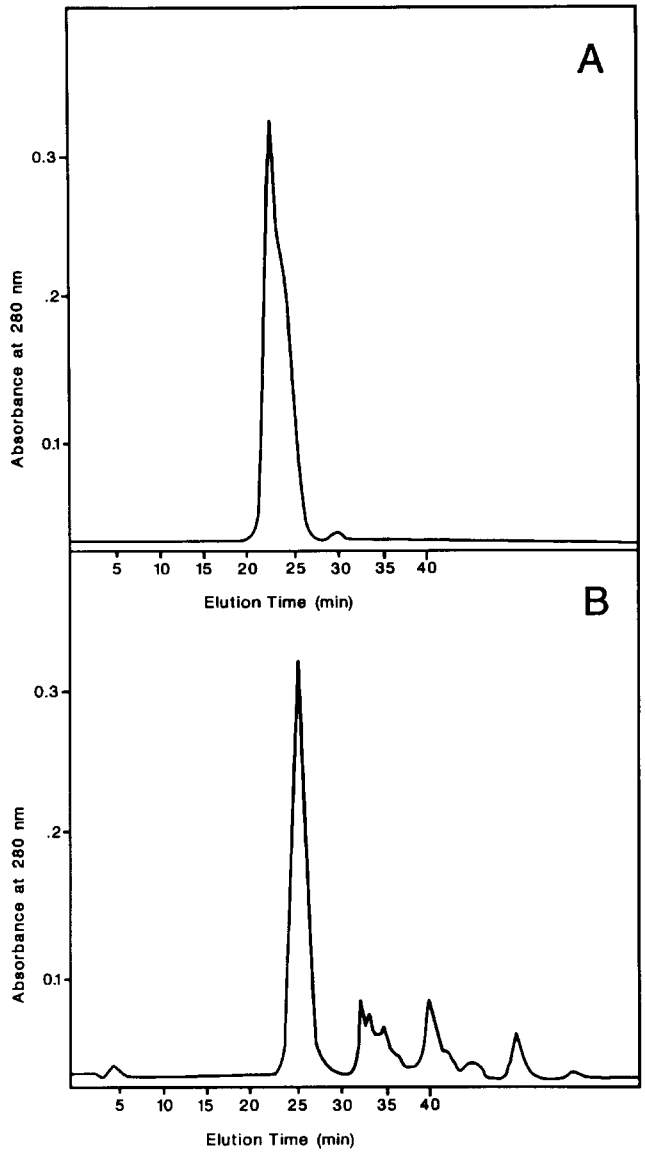


**Fig. 3.** FPLC gel filtration of *propep*·pepsin$_a$ and its acid-activation products. **A:** Complex *propep*·pepsin$_a$ (0.68 mg in 120 μL) from the MonoQ peak fraction was applied to an FPLC Superose 12 column, which was preequilibrated with 20 mM bis-Tris, 0.8 M urea, and 0.1 M NaCl, pH 6.0. The sample was eluted with the same buffer at a flow rate of 0.5 mL/min. **B:** The same amount of *propep*·pepsin$_a$ as in A was acidified to pH 2.0 by the addition of 20 μL of 0.5 M Na citrate, pH 2.0. The mixture was allowed to incubate at 20 °C for 10 min, and the insoluble aggregate was pelleted by microcentrifuge in an Eppendorf centrifuge for 8 min at 4 °C. The clear supernate was subjected to chromatography as in A. The protein peak was eluted at 25.7 min, compared to 22.2 min for the peak in A.

by acid activation of *propep·sin* (X. Lin et al., 1992). Pepsin$_a$ in the complex appears to have been degraded during acid activation, as its band has decreased to 33 kDa (Fig. 4, lane 4) from the untreated 40 kDa (Fig. 4, lane 3). These results indicated that, in an acid solution, the complex is activated and proteolytically cleaved in the

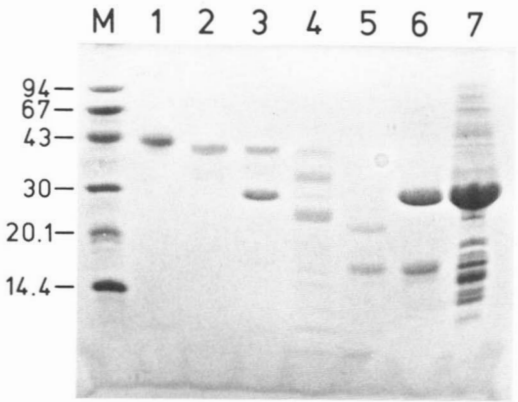


**Fig. 4.** SDS-PAGE electrophoresis patterns of *propep·pepsin* and its acid-activation products. Lane 1, pepsinogen (1 μg); lane 2, pepsin$_a$ (2 μg); lane 3, purified *propep*·pepsin$_a$ (2 μg); lane 4, acid-activated *propep*·pepsin$_a$ (2 μg); lane 5, acid-activated *propep·sin* (resulting in *pep·sin*, 1 μg); lane 6, untreated *propep·sin* (3 μg); lane 7, recombinant *propep* in cell lysate (5 μg) used as a marker for the position of *propep*.

*pro* region of the *propep* fragment and in the denatured *pep* region of pepsin$_a$.

### *Relative alkaline stability of propep·pepsin$_a$ to propep·sin, pepsin, and pepsinogen*

Complex *propep*·pepsin$_a$, two-chain pepsinogen, pepsin, and pepsinogen were compared for their stability in a 20-min incubation at different pH values ranging from 6.0 to 10.5. Figure 5 shows the well-known instability of pepsin above pH 6 and of pepsinogen above pH 10 (Fruton, 1971). Two-chain pepsinogen and complex *propep*·pepsin$_a$ are unstable above pH 8.5. The greater resistance to inactivation for *propep*·pepsin$_a$ in the pH range of 9–10 is the main difference between these two two-chain zymogens.

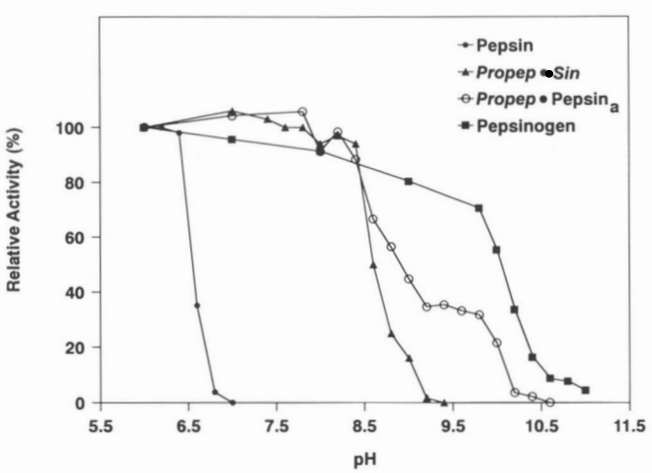


**Fig. 5.** Inactivation of *propep*·pepsin$_a$, *propep·sin*, pepsin, and pepsinogen in solutions of different pH values. The enzyme and zymogen solutions were preincubated in different pH solutions before assays.

### *Relative stability of propep, pep, and sin lobes at different pH values*

Pepsin$_a$ and the recombinant and individually folded lobes *propep* and *sin* were incubated separately in solutions of different pH values for defined time in order to find out their relative stability. The potentials for restoration of activity were assayed after the additions of recombinant *propep* (for preincubated pepsin$_a$ and *sin*) or *sin* (for preincubated *propep*). Figure 6 shows that recombinant *propep* loses its potential activity as pH increases from 6 to 11. On the other hand, recombinant *sin* loses partial activity only between pH 8 and 9, and does not lose further activity above pH 9. The loss of potential regeneration of activity of pepsin is similar to that of *sin* between pH 8 and 9, but further declines above pH 9. The difference between *sin* and pepsin above pH 9 is probably due to the ability of *sin* to refold in the activity-regenerating assay (at pH 6). In pepsin, the presence of denatured *pep* lobe may have prevented the refolding of its *sin* lobe. These results also confirm that *sin* is more alkaline stable, at least under the experimental conditions, than is *propep*.

## Discussion

The observed events from this study are schematically illustrated in Figure 7. The results indicate that the alkaline sensitivity of pepsin is due to the specific denatura-

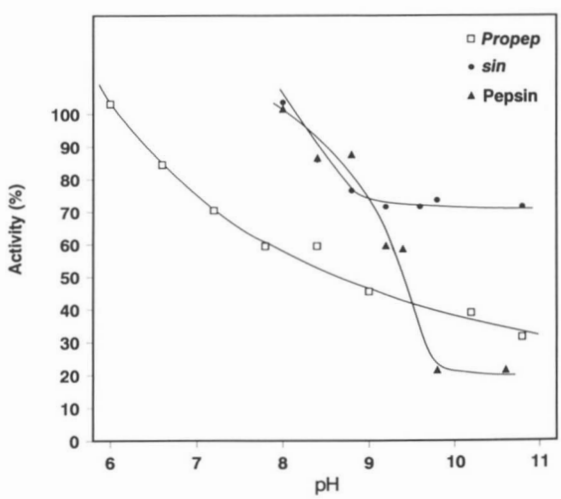


**Fig. 6.** Stability of the potential regenerating activity of *propep*, *sin*, and pepsin. Pepsin and recombinant *propep* and *sin* were preincubated individually in different pH solutions. Excess of recombinant *propep* was added to the preincubated pepsin and *sin*, while excess of *sin* was added to the preincubated *propep*. The activity of the solution was then assayed.

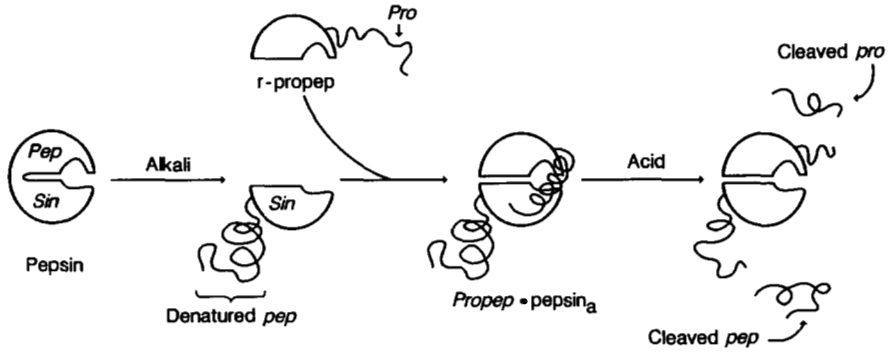


**Fig. 7.** Schematic presentation of observations made in this study. An alkaline solution denatures the *pep* lobe of pepsin to cause an inactivation. The addition of recombinant (r-) *propep* to the alkaline-denatured enzyme (pepsin$_a$) results in the formation of complex *propep*·pepsin$_a$, which resembles a two-chain pepsinogen (X. Lin et al., 1992). When the complex is in an acidic solution, the activation is accompanied by the cleavage of the *pro* peptide and the denatured *pep* domain of pepsin$_a$.

tion of its N-terminal lobe *propep*. This conclusion is supported by the regeneration of activity of the alkaline-inactivated pepsin by the addition of *propep* but not by *sin* (Fig. 1). The alkaline stabilities of the individual recombinant lobes (Fig. 6) also support this conclusion. Recombinant *sin* is stable at pH <8 but at high pH it may be in reversible denaturation (Fig. 6). It has long been known that pepsin undergoes irreversible denaturation above pH 6 (Fruton, 1971), which is independent of buffer used. Therefore, we consider the results obtained from the current studies to be a general model of alkaline pepsin denaturation.

The *propep*·pepsin$_a$ complex is sufficiently stable to purification. This complex spontaneously activates in acid with a concomitant cleavage in the *pro* region, illustrating that the added *propep* moiety and the lobe *sin* from the alkaline-treated pepsin must form a complex whose conformation is very near that of native pepsinogen (Fig. 7). During acid activation, at least two different types of cleavage take place. The first event is the cleavage of the *pro* peptide normally associated with the activation of pepsinogen. The fact that the activation does not reduce *propep* to the size of *pep* indicates that the observed activation product of *propep* (Fig. 4) is an activation intermediate. The incomplete cleavage of the *pro* peptide is apparently due to the much reduced rate of activation of this complex as compared to that of the native zymogen (see below). When porcine pepsinogen is activated in acid, the first bond to be cleaved is between P16 and P17 (Ile–Leu) in the *pro* region (Dykes & Kay, 1976). This is the probable site of the cleavage during activation of the complex. The second event is the digestion of the alkaline-denatured *pep* domain in pepsin$_a$ by the activated complex (Fig. 7). As a result, activation also produced a pepsin$_a$ fragment (Fig. 4). This is not surprising since the denaturation of *pep* domain in pepsin$_a$ exposes some hydrophobic residues normally buried in the tertiary structure of pepsin, thus providing specificity sites for cleavage. It is known that in acid the *pro* region is selectively "denatured" in pepsinogen; thus it is capable of substrate hydrolysis (Marciniszyn et al., 1976). The initial autodigestion of *propep*·pepsin$_a$ must have come from a similar mechanism. We have measured the activation kinetics of *propep*·pepsin$_a$ complex. The apparent first-order activation constant is at least 100 times slower (0.106 min$^{-1}$ in pH 2.0 at 29 °C) than that of the native pepsinogen or two-chain pepsinogen (results not shown). This is in part due to competition from the second proteolytic event (digestion of the denatured *pep* domain). This competitive inhibition is analogous to the inhibition of first-order activation of pepsinogen in the presence of hemoglobin substrate (Marciniszyn et al., 1976). A second factor for the slow activation rate is the hindrance of the denatured *pep* domain on the movement of the *pro* region, which is required in the activation mechanism (Marciniszyn et al., 1976).

In alkaline solution, complex *propep*·pepsin$_a$ is more stable than pepsin, less stable than pepsinogen, and similar in stability to two-chain pepsinogen (Fig. 5). The increased stability of *propep*·pepsin$_a$ in the pH range of 9–10, however, is somewhat surprising. This is possibly due to the nonspecific interaction between the denatured *pep* domain in pepsin$_a$ with the added recombinant *propep*, which may have slowed the rate of denaturation.

We have used the known three-dimensional structure of pepsin to generate information on the groups that may be responsible for the alkaline denaturation of the N-terminal lobe of pepsin (Kinemage 2). Pepsin is a very acidic protein with an isoelectric point below pH 1 (Tiselius et al., 1938). This can be attributed to a large number of negatively charged groups, 23 in the *pep* domain and 19 in the *sin* domain. The only two positive groups in the *pep* lobe are the $\alpha$-amino group at the N-terminus and His$^{57}$. Both of these groups are located on the outside of the three-dimensional structure. Thus the loss of charges of these groups above their p$K_a$ values is unlikely to cause the denaturation of the *pep* lobe. Therefore, the most reasonable explanation of the denaturation of the *pep* domain above pH 7 is the ionization of some carboxyl groups with p$K_a$ values in the neutral and alkaline range. Normally, carboxyl groups of aspartic and glutamic side chains have p$K_a$ values near pH 4. However, in a special environment, such as buried in the tertiary structure of the protein or interacting with other charged groups, the p$K_a$ of these carboxyl groups can be greatly elevated. Figure 8 and Kinemage 2 show all residues in pepsin with partially or

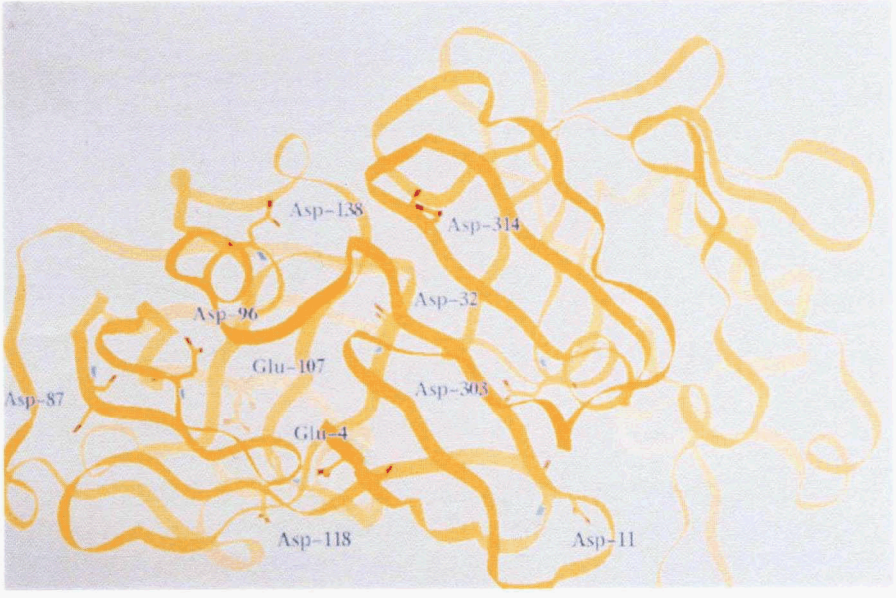


**Fig. 8.** Ribbon model of pepsin with residues potentially important for alkaline denaturation of the enzyme. Superimposed on the ribbon view of pepsin are the acidic titratable side chains (with residue numbers), which are not fully exposed to the solvent and hence could have abnormal p$K_a$ values. Criteria for solvent exposure were taken from the structural work of Sielecki et al. (1990). Eight out of the 10 residues marked in the figure are located in the N-terminal lobe (residues 1–172). These groups could contribute to the pH-dependent denaturation of the N-terminal lobe.

completely buried carboxyl groups. It can be seen that most of these residues are located in the N-terminal lobe, ***pep*** (residues 1–172). Thus they are potential contributors to the alkaline denaturation. In order to investigate the effects of closely positioned carboxyls on their ionizations, we have used Mehler distance dielectric dependence (Mehler & Eichele, 1984) to calculate the electrostatic potentials of these groups based on their interaction with other negatively charged groups in the vicinity. As shown in Table 1, Asp'' and $Asp^{159}$ are among several with the largest potential observed. It is interesting that Asp'' is partially buried and forms a hydrogen-bonded dyad with $Asp^{159}$, also located in the N-terminal lobe (Fig. 9; Kinemage 2). This relationship is analogous to that of the active-site $Asp^{32}$ and $Asp^{215}$. The former also has a high electrostatic potential (Table 1). We have reported that the p$K_a$ values of the active-site dyad are 1.57 and 5.02 (Y. Lin et al., 1992) in native pepsin. Because Asp'' is much more buried than the active-site residues, its p$K_a$ is likely to be higher than ***5***. Characteristically, the counterpart of the dyad, $Asp^{159}$, may have a p$K_a$ lower than Asp''. Thus, the ionization of Asp'' at neutral or alkaline solutions would place two negative charges in close vicinity. This is an unfavorable interaction for these two aspartyl groups and may play an important role in the denaturation of the ***pep*** domain in pepsin. Other residues with high electrostatic potentials (Table 1) that may be important in the alkaline denaturation of the N-terminal lobe are $Glu^4$, $Glu^{13}$, and $Asp^{118}$, all high in electrostatic potentials (Table 1). All these groups are buried in the tertiary structure of pepsin. Thus, their ionization may induce conformational changes in order for these side chains to gain access to and be stabilized in an aqueous environment. The role of these residues in pepsin denaturation in alkaline solutions may be tested in future experiments using site-directed mutagenesis.

## Materials and methods

### *Materials*

Recombinant ***propep*** and ***sin,*** purified and refolded, are prepared as previously described (X. Lin et al., 1992). Porcine pepsin was purchased from Sigma. Other reagents are the highest quality obtained commercially.

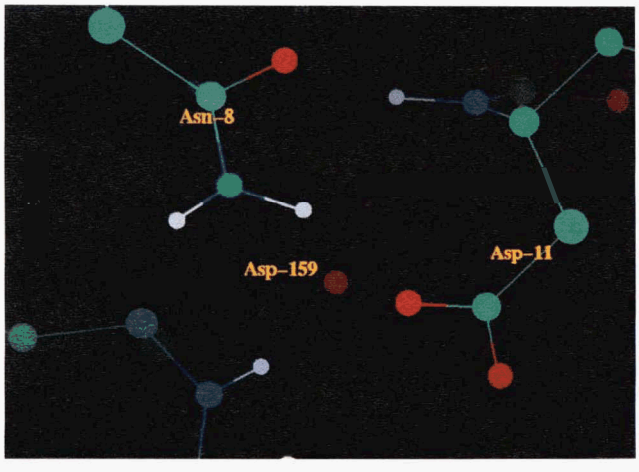


**Fig. 9.** The environment of residues Asp'' and $Asp^{159}$. Asp'' and $Asp^{159}$ form a hydrogen bond dyad similar to that of the active-site $Asp^{32}$ and $Asp^{215}$. Asp'' is only partially accessible to the solvent. It is hypothesized that $Asp^{159}$ and Asp'', respectively, have abnormally low and high p$K_a$ values for carboxyl groups. The ionization of both groups in neutral and alkaline solutions in close vicinity is disruptive to the conformation of the N-terminal lobe. Other groups that may modulate the p$K_a$ values of this dyad are $Asn^8$, which forms a hydrogen bond with Asp'', and the backbone peptide amide group of $Asp^{159}$, which forms a hydrogen bond with its side chain. Carbon, oxygen, and hydrogen atoms are shown in green, red, and white, respectively.

**Table 1.** *Electrostatic potential on acidic residues of the N-terminal (pep) lobe of pepsin*

| Residue | Potential energy (kcal/mol) |
|---|---|
| Asp 3 | −3.7 |
| Glu 4 | −5.5 |
| Glu 7 | −4.9 |
| Asp 11 | −6.3 |
| Glu 13 | −5.5 |
| Asp 26 | −3.7 |
| Asp 32 | −6.5 |
| Asp 52 | −3.0 |
| Asp 59 | −3.8 |
| Asp 60 | −4.4 |
| Glu 65 | −2.5 |
| Glu 70 | −1.0 |
| Asp 87 | −4.2 |
| Asp 96 | −3.7 |
| Glu 105 | −2.7 |
| Glu 107 | −4.5 |
| Asp 118 | −5.4 |
| Asp 138 | −0.6 |
| Asp 142 | −3.7 |
| Asp 149 | −2.8 |
| Asp 159 | −8.0 |
| Asp 160 | −3.8 |
| Asp 171 | −2.1 |

### *Buffers*

Buffers used for pH-dependent experiments included buffer A, pH 6–9.6, 50 mM potassium phosphate, 50 mM bis-Tris, 50 mM Tris, and 100 mM NaCl, and buffer B, pH 9.8–11.0, 50 mM cyclohexylaminopropane sulfonic acid in buffer A.

### *Proteolytic assays*

Proteolytic activity was assayed using either radiolabeled bovine hemoglobin substrate (Lin et al., 1989) or synthetic peptide KPAEFF($NO_2$)AL, (F($NO_2$) is *p*-nitrophenylalanine), as described previously (Fusek et al., 1990).

### *Purification of the complex of propep to alkaline-inactivated pepsin (propep·pepsin$_a$)*

Pepsin (150 mg) was dissolved in 4 L of 20 mM Tris-HCl, pH 9.0. This solution was kept at 20 °C for 30 min to completely inactivate the pepsin. A 400-mL urea-solubilized recombinant *propep* solution (X. Lin et al., 1992) was then diluted dropwise into the inactive pepsin solution with rigorous stirring. The final solution was kept at 20 °C for 15 min, and then 80 mL of 1 M bis-Tris, pH 6.0, was added slowly with stirring. After adjusting the pH to 6.2 with 1 M HCl, this solution was kept overnight at 4 °C. The subsequent purification procedures for active complex of *propep*·pepsin$_a$ was the same as described previously for the purification of *propep·sin* (X. Lin et al., 1992).

### *pH stability of proteolytic activity*

Ten microliters of a solution containing either pepsinogen, *propep·sin*, *propep*·pepsin$_a$ (in 20 mM Tris-HCl, pH 8.0), or pepsin (in 20 mM bis-Tris, pH 6.0) was added to a series of 40 μL of buffers (buffer A or B) ranging in pH from 6.0 to 10.5. The solution was left standing at 20 °C for 20 min and then added to a premixed solution containing 50 μL of synthetic substrate (KPAEFF($NO_2$)AL, 2 mg/mL), 110 μL of $H_2O$, and 1 mL of 0.2 M sodium citrate, pH 2.0, preequilibrated at 37 °C. The hydrolysis rate was monitored at $A_{300nm}$ in an HP8452A diode array spectrophotometer, and the initial rate was taken as the enzyme activity.

### *pH stability of the potential regenerating activity*

Recombinant fragments *propep* and *sin* were refolded at pH 6 and 8, respectively, as previously described (X. Lin et al., 1992). Alkaline-inactivated pepsin, pepsin$_a$, was prepared by direct dissolving of pepsin in a 20 mM Tris-HCl buffer, pH 8.0.

#### *Stability of propep*

To 10-μL aliquots of *propep* solution (10 mg/mL in 20 mM bis-Tris, pH 6), 90 μL of buffer of different pH values were added and incubated at 20 °C for 20 min. Aliquots of 20 μL of 1 M bis-Tris, pH 6, and 2 μL of *sin* solution (7 mg/mL in 20 mM Tris-HCl, pH 8.0) were added separately. The samples were assayed at pH 2 using $^{14}$C-hemoglobin as substrate (Lin et al., 1989).

#### *Stability of sin*

Aliquots of *sin* solution (as for *propep*) were diluted five times individually with buffers of different pH values and incubated at 20 °C for 20 min. Two-microliter aliquots of this solution were mixed separately with 1 μL of 1 M bis-Tris, pH 6, and then 37 μL of a *propep* solution (as for *propep*). After incubation at 20 °C for 10 min, the samples were assayed as for *propep*.

#### *Stability of pepsin$_a$*

Aliquots of pepsin$_a$ solution were diluted individually 200-fold with buffers of different pH values and incubated at 20 °C for 20 min. Two microliters of each sample were treated in the same manner as *sin* (above).

### *Calculation of electrostatic potential energies*

To determine the interaction energies among charges we employed a simple coulombic expression with a sinusoidal dielectric constant introduced by Mehler and Eichele

(1984). In this approach, the shift in $pK_a$ due to the presence of other charges is given by

$$\Delta pK_a^{(i)} = (2.3K_bT)^{-1}q_i\Phi_i,$$

where $K_b$ is Boltzmann's constant, $T$ is temperature, and $\Phi_i$ is the potential at charge $i$,

$$\Phi_i = \sum_j q_j/\epsilon_{ij}R_{ij}.$$

Here $R_{ij}$ is the distance between charges $q_i$ and $q_j$, and $\epsilon_{ij}$ is the sinusoidal distance-dependent dielectric, which is given by

$$\epsilon_{ij}(R_{ij}) = A + B/[1 + k\exp(-\lambda BR_{ij})],$$

where $B = \epsilon_{H_2O} - A$, $A = -20.9$, $k = 3.5$, and $\lambda = 0.0018$. The dielectric constant of water is $\epsilon_{H_2O}$. All basic and acidic titratable groups are taken into account in the calculation.

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