Mechanism of Intramolecular Activation of Pepsinogen

EVIDENCE FOR AN INTERMEDIATE δ AND THE INVOLVEMENT OF THE ACTIVE SITE OF PEPsin IN THE INTRAMOLECULAR ACTIVATION OF PEPSINOGEN*

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JOSEPH MARCINSZYN, JR., JUNG SAN HUANG, JEAN A. HARTSUCK, AND JORDAN TANG

From the Laboratory of Protein Studies, Oklahoma Medical Research Foundation, and the Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Intramolecular pepsinogen activation is inhibited either by pepstatin, a potent pepsin inhibitor, or by purified globin from hemoglobin, a good pepsin substrate. Also, pepsinogen at pH 2 can be bound to a pepstatin-Sepharose column and recovered as nativezymogen upon elution in pH 8 buffer. Kinetic studies of the globin inhibition of pepsinogen activation show that globin binds to a pepsinogen intermediate. This interaction gives rise to competitive inhibition of intramolecular pepsinogen activation. The evidence presented in this paper suggests that pepsinogen is converted rapidly upon acidification to the pepsinogen intermediate δ. In the absence of an inhibitor, the intermediate undergoes conformational change to bind the activation peptide portion of this same pepsinogen molecule in the active center to form an intramolecular enzyme-substrate complex (intermediate θ). This is followed by the intramolecular hydrolysis of the peptide bond between residues 44 and 45 of the pepsinogen molecule and the dissociation of the activation peptide from the pepsin. Intermediate δ apparently does not activate another pepsinogen molecule via an intermolecular process. Neither does intermediate δ hydrolyze globin substrate.

Several recent studies have established that porcine pepsinogen may be activated by either of two main mechanisms, an intramolecular activation mechanism or a pepsin-catalyzed activation process. The intramolecular activation, which has been demonstrated independently with different techniques in three laboratories (1-3), proceeds by first order kinetics; consequently, its activation rate constant is independent of pepsinogen concentration (2). The chemical event which is characteristic of intramolecular activation is the hydrolysis of a peptide bond between residues 44 and 45 (Leu-Ile) of pepsinogen to form the NH₂ terminus of pepsin (4). Since the magnitude and pH dependence of the kinetic constants for intramolecular activation are similar to the kinetic properties of pepsin hydrolysis we have postulated that intramolecular activation takes place at the same active site as is responsible for the pepsin hydrolysis of substrates (2).

In this paper we report more detailed studies of the mechanism of intramolecular activation. By using a substrate and an inhibitor of pepsin, we have established the presence of a previously unproven intermediate in the intramolecular activation of pepsinogen. The properties of this intermediate are described. The mechanistic implications of the existence of this intermediate are discussed.

EXPERIMENTAL PROCEDURE

Materials

Chromatographically pure porcine pepsinogen (Lot FGC55J413) was purchased from Worthington Biochemical Corp. Some of the pepsinogen used was purified in our laboratory as described previously (2). Sulfoethyl (SE)-Sephadex C-25 and other Sephadex gels were obtained from Pharmacia. The chemicals and solvents used for the Protein Sequencer 890C were the products of Beckman Instruments. Bovine hemoglobin was purchased from Pentex. For some experiments, the heme was removed and the resulting globin was purified as previously described (5). Throughout this paper the term globin is used for this protein preparation. Pepstatin was a gift of Drs. Umezawa and Aoyagi, Institute of Microbial Chemistry, Tokyo, Japan. All other reagents were of the highest purity available commercially.

Proteolytic Activity Assay

Proteolytic activity toward a bovine hemoglobin substrate was measured using a modification of the method of Anson and Mirsky (6, 7).

Polyacrylamide Disc Gel Electrophoresis

Disc gel electrophoresis was performed as described by Davis (8) with 7% acrylamide gels in Tris/glycine buffer, pH 8.3. Protein samples of about 10 µg were mixed with the sample gel solution. Electrophoresis
Pepsinogen Activation Mechanism

Two methods were used to follow the pepsinogen activation in the presence of globin.

**Assay for Pepsinogen Remaining in Activated Mixture**—The general scheme for these experiments was the same as that used earlier (2) except that the activation mixture contained globin. Conditions and procedures for a typical experiment at a pepsinogen concentration of 2 x 10^-4 M, a globin concentration of 1% and pH of 2.0 were as follows. Pepsinogen (6.5 mg) was dissolved in 5 ml of glass-distilled water to give 3.1 x 10^-5 M pepsinogen, pH 5.3. Then 5 µl of this solution were added to 150 µl of 1.67 x 10^-5 M (i.e. 1.03%) globin, pH 2.0. This globin solution was equilibrated in a 37° bath. After an appropriate time interval, which was measured with a stop watch, 125 µl of 0.5 M Tris/chloride, pH 8.5, were added. The additions of the zymogen and the basic buffer both were made with syringes and were as instantaneous as possible. The process was repeated for each predetermined time interval (i.e. 5, 10, 15 s, etc.) at each globin concentration. After at least 20 min at alkaline pH, the entire volume of each solution was assayed for remaining pepsinogen activity as described below.

The potential proteolytic activity was assayed at 37° with globin as substrate. To the 0.28 ml of alkaline pepsinogen solution, 0.72 ml of a solution containing 0.55 M citric acid and 0.10 M HCl were added in order to adjust the pH to 2.0. The remaining pepsinogen was allowed to activate for 30 min. One milliliter of 2.5% globin solution, pH 2.0, was added to each tube; then, after 20 min, 1 ml of 10% trichloroacetic acid solution was added. The mixture was filtered on Whatman No. 50 filter paper and the optical density of the filtrate was read at 280 nm against a blank to which 1 ml of 10% trichloroacetic acid solution was added prior to the addition of the 1 ml of globin substrate solution. Use of this blank allowed quantification of the pepsinogen remaining after the initial activation because optical density due to soluble peptides produced during the two activation periods was included appropriately in the blank. In all assay situations, the net optical density at 280 nm was 1.2 or less, and the blank did not exceed 0.75 optical density.

**Measurement of Appearance of Proteolytic Activity by pH-stat Assay**—The method was the same as that described in a separate work (5) except that pepsinogen rather than pepsin was added to the globin solution. In a typical experiment, 0.01 ml of 1.62 x 10^-4 M pepsinogen was added to about 1.8 ml of 1.6 x 10^-4 M globin solution at pH 2 and 37°. The

**Activation of Pepsinogen in Presence of Globin**

Two methods were used to follow the pepsinogen activation in the presence of globin.

**Scheme 1**

\[ \text{Pepsinogen} \xrightarrow{k_{1}} \text{Intermediate} S \xrightarrow{k_{2}} \text{Pep} \alpha + \text{peptides} \]

**Scheme 2**

\[ \text{n} \xrightarrow{k_{1}} \alpha + \text{peptides} \]

**Scheme 3**

\[ \text{s} \xrightarrow{k_{1}} \alpha + \text{peptides} \]

**Quantitative Determination of Pepsinogen and Pepsin in Activation Mixture**

Determination of pepsinogen and pepsin was carried out by two methods. First, the ratio of the two proteins in a mixture was determined from the ratio of phenylthiohydantoin-valine and phenylthiohydantoin-glycine in the second cycle of an automated Edman degradation. The procedures and accuracy of this determination have been described previously (4). Second, the pepsinogen activity was assayed independent of pepsin after alkaline inactivation of the latter. The details of this method also have been published (2).
resulting pepsinogen concentration was $1 \times 10^{-4}$ M. The consumption of 0.04 M HCl as a function of time was recorded. These experimental curves had a gradually increasing slope and ultimately attained a constant maximal velocity. The slope of the tangent to these curves was deemed proportional to the pepsinogen concentration at that time. Maximum velocity was the same as that generated by a preactivated pepsinogen sample of the same concentration. The ratio of the slope at a given time to the final maximal slope was multiplied by the initial pepsinogen concentration to yield the pepsin concentration.

**Kinetic Treatments**

Scheme 1 in Fig. 1 shows the reactions which we have used to fit our observations of the inhibition of pepsinogen activation by globin. Justification of the validity of this scheme is included under “Results.” The rate equation for Scheme 1 is

$$\frac{d[\alpha]}{dt} = k_{1st}[\Theta]$$

(1)

where $\alpha$, $\Theta$, and $k_{1st}$ are defined in Scheme 1 (Fig. 1) and $t$ is time. Also from Scheme 1

$$K_c = \frac{[\Theta]}{[\Theta]}$$

(2)

$$K_{IG} = \frac{[\delta \cdot G]}{[\delta \cdot G]}$$

(3)

$$[P_1] = [\delta] + [\delta \cdot G] + [G] + [G]$$

(4)

where $K_{c}$, $\alpha$, $\Theta$, $G$, and $\delta$ are defined in Scheme 1 (Fig. 1), brackets denote concentration of these species, and $[P_1]$ is the initial concentration of pepsinogen which is equal to the total concentration of all forms of pepsinogen. (Initial pepsin concentration is zero.)

Substitution of Equations 2 and 3 into Equation 1 followed by graphical similarity to the schematic characteristics of the intermediates described by the constants $K_{c}$ and $K_{e}$ is rapid with respect to the activation described by $k_{1st}$. Also, the globin concentration is considered constant during the experiment. These assumptions are justified under “Results.” Rearrangement of Equation 6 gives

$$\ln \left( \frac{[P_1] - [\alpha]}{[P_1]} \right) = -At$$

(5)

where

$$\Lambda = \frac{k_{1st}G}{K_c \cdot K_{IG}}$$

(6)

and all other symbols are as defined above. In this treatment it is assumed that the conversion of $\alpha$ to $\Delta$ and establishment of the equilibria described by the constants $K_{c}$ and $K_{e}$ are rapid with respect to the activation described by $k_{1st}$. Also, the globin concentration is considered constant during the experiment. These assumptions are justified under “Results.” Rearrangement of Equation 6 gives

$$\frac{1}{\Lambda} = \frac{[G]}{k_{1st} K_c K_{IG}} + \frac{1}{k_{1st} K_c} \left( 1 + \frac{1}{K_c} \right)$$

(7)

Therefore a plot of the reciprocal of the pepsinogen decay constant $A$ versus globin concentration should be linear and have an intercept whose reciprocal is equal to the pepsinogen decay constant in the absence of globin. Since in this treatment, the $\delta \cdot G$ complex is a dead-end, the globin inhibition of the activation reaction is analogous to competitive enzyme inhibition.

Scheme 2 of Fig. 1 has been considered as an alternative. In this case, intermediate $\delta$ is not obligatory. If the conformational equilibria between the various pepsinogen species are established rapidly as compared to the hydrolysis of the peptide bond to form pepsin, then we can not distinguish with our measurements between the obligatory and the nonobligatory mechanisms (see “Discussion”).

A more complicated system in which the pepsinogen-globin complex ($\delta \cdot G$) can be transformed to pepsin ($\alpha$) has been considered (see Scheme 3 in Fig. 1). An integrated rate expression analogous to Equation 4 but the decay constant is

$$A' = \frac{k_{1st} K_c G + k_{1st} [G] + K_{IG} + [G]}{K_{IG} + [G]}$$

(8)

where $A'$ is the decay constant, $k_{1st}$ is first order rate constant for transformation of $\delta \cdot G$ to $\alpha$, and the other symbols are the same as above. Rearrangement of Equation 8 shows that in this instance the plot of $1/A'$ versus $G$ is not linear but is a curve which approaches a maximum, which is $1/k_{1st}$, as $G$ is increased. Here because the $\delta \cdot G$ complex is productive, albeit at a different rate, the inhibition is somewhat analogous to noncompetitive enzyme inhibition.

**RESULTS**

**Inhibition of Pepsinogen Activation by Pepstatin—Activation of 0.05% pepsinogen in the presence of about a 6-fold molar excess of pepstatin was almost completely inhibited (Table I). However, it was necessary to include 5% methanol in the zymogen solution to completely dissolve the pepstatin. Although the concentration of methanol used has been shown by Neuman and Shinitzky (13) to produce no inactivation of pepsinogen, a set of three experiments was carried out in the absence of methanol. In these experiments (Nos. 3, 4, and 5 in Table I), only about a 4-fold molar ratio of pepstatin to pepsinogen was used due to the low solubility of the inhibitor. As shown in Table I, a definite inhibition of pepsinogen activation by pepstatin also existed under these conditions.**

**Binding of Pepsinogen Intermediate to Pepstatin/Sephose Column—** Since pepsinogen activation is inhibited by pepstatin, we devised the following experiments to verify the binding of a pepsinogen intermediate to a pepstatin/Sephose column. Fig. 2a shows that pepsinogen at pH 5.6 in 1 M NaCl was not retained by a pepstatin/Sephase column and thus emerged in a breakthrough peak. Pepsin under the same conditions was retained and could be eluted from the column only as alkaline inactivated enzyme in pH 8.5 (Fig. 2b). However, pepsinogen was absorbed on the pepstatin/Sephase immediately after being acidified to pH 2.2. (There was no significant breakthrough peak, Fig. 2c.) A peak was eluted in pH 8, 0.05 M sodium phosphate buffer (Fig. 2c). This peak, which represented about 30% of the original zymogen, had alkaline stability in pH 8 and had the same specific activity as the starting pepsinogen. The amino acid composition, NH$_2$-terminal 2-residue sequence, and the mobility on polyacrylamide disc gel electrophoresis (Fig. 3) were identical to the native pepsinogen. Since the recovered pepsinogen was not eluted in pH 5.6 buffer with 1 M NaCl, the zymogen must have been absorbed on the affinity column as an intermediate which reverted to native pepsinogen at pH 8.

A second peak was eluted from the affinity column with pH 8 buffer and 1 M NaCl. This sample had an amino acid...
Inhibition of pepsinogen activation by pepstatin

| Experiment | Components | Peptatin/pepsinogen molar ratios | Methanol | Temperature | Time | Procedure for removing peptides | Pepsinogen* remaining | Ile-Gly-Pepsin* produced |
|------------|------------|---------------------------------|----------|-------------|------|---------------------------------|----------------------|------------------------|
| 1          | Pepsinogen | 6                               | +        | 14°C        | 120 min | Dialysis                        | 35.7                 | 64.3                   |
| 2          | Pepsinogen + pepstatin | 6 | + | 14°C | 60 min | Dialysis | 94.4 | 5.6 |
| 3          | Pepsinogen | 4 | - | 14°C | 75 min | Sephadex G-25 | 71.4 | 28.6 |
| 4          | Pepsinogen + pepstatin | 4 | - | 10°C | 60 min | Sephadex G-25 | 96.8 | 3.2 |
| 5          | Pepsinogen | 4 | - | 11°C | 75 min | SE-Sephadex | 84.9 | 16.0 |

*The pepsinogen used in Experiments 1 to 3 was a Worthington product. Pepsinogen used in Experiments 4 and 5 was purified according to Al-Janabi et al. (2).

*The percentages of pepsinogen remaining and Ile-Gly-pepsin produced were calculated from the quantitation of phenylthiohydantoins (see "Methods") based on the assumption that Ile-Gly-pepsin was essentially the only product of pepsinogen activation under the experimental conditions (4).

Fig. 2. Elution patterns on an affinity column of pepstatin/aminohexyl-Sepharose 4B. Each fraction contains 3 ml of effluent. The solid line and the dotted line are, respectively, the protein concentration and proteolytic activity. A, pepsinogen in 1 M sodium acetate buffer, pH 5.6, with 1 M NaCl was not retained by the affinity column and emerged in the breakthrough peak. B, pepsin in 1 M sodium acetate buffer, pH 5.6, with 1 M NaCl was retained by the column and was eluted in 0.05 M sodium phosphate buffer, pH 8.5, as alkaline inactivated pepsin; the smaller breakthrough peak represents some inactive pepsin in the commercial preparation. C, pepsinogen in 0.1 M glycine/HCl buffer, pH 2.2, was retained by the affinity column and could not be recovered by eluting with 0.1 M sodium acetate buffer, pH 5.6, with 1 M NaCl. Two peaks were eluted subsequently with 0.01 M sodium phosphate buffer, pH 8, and the same buffer containing 1 M NaCl.

Fig. 3. Polyacrylamide disc gel electrophoretic patterns of samples from pepstatin/Sepharose column chromatography. The samples are: left, native pepsinogen; center, material eluted in pH 8 buffer (first peak in Fig. 2); right, material eluted in pH 8 buffer with 1 M NaCl (second peak, Fig. 2). The major band in this gel represents the denatured pepsinogen α (see text). A very light band slightly behind the major band is denatured pepsin. The mobility of about 85% of the native pepsinogen (Fig. 3). In addition, two minor bands with mobilities corresponding to those of alkaline denatured pepsin and native pepsinogen were present. When an unstained gel was sectioned, eluted, and assayed after electrophoresis, all the proteolytic activity was found in the native pepsinogen position. The alkaline-denatured pepsin must have been derived from a small amount of activation during mixing with the pepstatin/Sepharose. Native pepsinogen in the second peak was probably the result of "tailing" from the first peak. The major material, the heavy band in the third gel of Fig. 3, had no potential...
proteolytic activity and must be an inactivated pepsinogen. (We will call this species pepsinogen \( \sigma \)). From the results described above, it can be concluded that, at pH 2, an intermediate form of pepsinogen was generated and bound to the pepstatin affinity column. In pH 8 buffer, only about 30% of the bound zymogen was recovered as native pepsinogen. About 70% of the bound zymogen was recovered as inactive pepsinogen \( \sigma \). It is important to point out that the half-life of pepsinogen activation at pH 2, 28\(^{\circ}C \), is about 16 s (2). Even at a lower temperature, a large fraction of zymogen is activated within 2 h (see Table I). Therefore, inhibition of pepsinogen activation by Sepharose-bound pepstatin must have taken place.

It is interesting that pepsinogen \( \sigma \), forms a single, well-defined band in polyacrylamide electrophoresis. In contrast, alkaline or alcohol denatured pepsinogen each produced six or seven bands under the same electrophoretic conditions. This comparison suggests that inactive pepsinogen \( \sigma \) assumed a rather uniform conformation which may resemble the conformation of the bound intermediate. A dialyzed pepsinogen \( \sigma \) fraction was completely absorbed again on the pepstatin/Sepharose column and was recovered by elution with pH 8 buffer, 1 mM NaCl. Attempts to regenerate potential proteolytic activity from pepsinogen \( \sigma \) by dialysis in pH 8, 4\(^{\circ}C \), were not successful.

**Effect of Globin on Pepsinogen Activation Rate**—From the above experiments we deduced that a low pH form of pepsinogen is capable of binding pepstatin, a potent pepstatin inhibitor. We then studied the activation of pepsinogen in the presence of high concentrations of globin, a good protein substrate for pepsin. Pepsinogen, 1 x 10\(^{-5}\) M, was incubated at pH 2 in the presence of globin, whose concentration ranged from 1 x 10\(^{-5}\) to 28.41 x 10\(^{-5}\) M. Semilogarithmic plots of the per cent of pepsinogen remaining versus incubation time were linear (Fig. 4). All experiments except the slowest one were followed until at least one-half of the pepsinogen had been activated. The linearity of these plots gave credence to the assumption that the bond cleavage reaction described by \( k_{nat} \) was much slower than either of the conformational changes or the binding of globin to pepsinogen \( \delta \). Increasing globin concentration caused a dramatic decrease in the apparent first order activation rate constant (Table II). The reciprocal plot defined by Equation 6 is quite linear (Fig. 5). The linearity of the plot over a 20-fold change in globin concentration supports the reversible, nonproductive, competitive globin inhibition contained in Schemes 1 or 2 of Fig. 1. In the 1/A \( G \) plot, the intercept divided by the slope of this line is 1.71 x 10\(^{-5}\) M and should be equal to \( K_{ia}(K_a + 1) \). Unfortunately, we cannot calculate values of the constants \( K_{ia} \) and \( K_a \) from this data. However, as described above, the inhibition of pepsinogen intramolecular activation by globin is “competitive,” which implies that the pepsin active site is bound to globin and therefore cannot catalyze intramolecular pepsinogen activation. We would expect globin binding to pepsinogen to be similar to but no tighter than globin binding to pepsin. Consequently, \( K_{ia} \) would not be smaller than \( K_a \) for globin as a pepstatin substrate. Our measured value of this \( K_a \) is 1.41 x 10\(^{-5}\) M (5). This implies that \( K_a \) is less than 0.1; i.e. the ratio of the concentrations of intermediates \( \delta \) and \( \theta \) is 10:1 or greater. The maximum possible value of \( K_{ia} \) is 1.7 x 10\(^{-8}\) M.

An alternative reaction scheme (Scheme 3, Fig. 1) has been considered and has been rejected because its predicted kinetics are not in agreement with our observations. This scheme where

![Diagram of pepsinogen activation](http://www.jbc.org/)

**Table II**

| Experiment | Globin concentration A\(^a\) | \( K_m(K_a + 1)^b \) | K(\( K_a + 1)^b \) |
|------------|-------------------------------|---------------------|-------------------|
| 1          | 0                             | 2.45                |                   |
| 2          | 1.56                          | 1.09                | 1.27              |
| 3          | 3.42                          | 0.78                | 1.62              |
| 4          | 7.11                          | 0.46                | 1.66              |
| 5          | 16.16                         | 0.25                | 1.85              |
| 6          | 28.41                         | 0.17                | 2.13              |
| Average    |                               |                     | 1.71              |

\( ^a \) Apparent first order activation rate constant, see Equation 6 under "Methods."

\( ^b \) Inhibition constant for globin's competitive inhibition of the pepsinogen activation.
the 1/A versus G plot. If this were interpreted to be due to production of pepsin from the δ-G complex, the rate constant for the process (k′act) would be less than 1/δ of kact. Consequently the contribution of the alternate pathway would be insignificant. Another reasonable explanation for the curvature is slight insolubility of globin at the higher concentrations.

The rate of conversion of pepsinogen to pepsin in the presence of globin was also determined in a pH-stat assay. Fig. 6 shows the semilogarithmic plot derived from the pH-stat experiments for 1 × 10⁻⁵ M pepsinogen and 1.61 × 10⁻⁵ M globin. The pepsinogen decay constant A was 0.23 min⁻¹. This value is in very good agreement with the proteolytic activity assay results shown in Table II for 16.16 × 10⁻⁵ M globin, which has a value of 0.25 min⁻¹ for A. This agreement not only substantiates the validity of the experiments described above but also indicates that the pepsinogen does not possess significant proteolytic capability toward globin substrate. (In Fig. 6, the zero time intercept of 90% rather than 100% pepsinogen may indicate some proteolytic capability for a pepsinogen species.) Total acid consumption in these assays corresponded to two to three cleavages per globin molecule. Therefore, the assumption in the kinetic treatment of constant globin inhibitor concentration during an experiment is justified.

**DISCUSSION**

Our current understanding of intramolecular pepsinogen activation is summarized in Fig. 7 and diagrammatically in Fig. 8. Native pepsinogen and active pepsin are referred to as n and α, respectively. Intermediate δ is the pepsinogen species which binds the inhibitors pepstatin and globin. Intermediate θ has the activation peptide in the active site. The species φ is the pepsin-peptide complex after cleavage but before dissociation of the peptide. The inactive pepsinogen which is recovered from the pepstatin/Sepharose column is called σ.

Two lines of evidence for the existence of intermediate δ have been obtained. First, pepstatin at pH 2, either in solution or attached to an affinity column, binds to pepsinogen and greatly retards pepsinogen activation (Table II and Fig. 2c). This interaction implies that at pH 2 pepsinogen assumes a conformation and a state of ionization capable of binding pepstatin and that this pepsinogen pepstatin complex is nonproductive, i.e. cannot be converted to pepsin. The confirmation of the pepsinogen species which binds pepstatin, intermediate δ, must differ from that of the native zymogen. The affinities of the native pepsinogen and intermediate δ toward the pepstatin/Sepharose column are strikingly different (Fig. 2). Second, the globin inhibition of pepsinogen activation indicates that a pepsinogen/globin complex forms. This pepsinogen/globin interaction and its inhibition of intramolecular pepsinogen activation is analogous to competitive inhibition of an enzyme/substrate reaction. These results are consistent with the formation of an intermediate δ.

Because our kinetic data fit the equations derived from Schemes 1 or 2 (Fig. 1), we conclude that the pepsin substrate globin binds reversibly to a pepsinogen intermediate called δ. The kinetic data also indicate that the δ-globin complex cannot be converted to pepsin. As shown diagrammatically in Fig. 8, the intermediate is thought to have a developed active site which can bind either a pepsin inhibitor, pepstatin, or a pepsin substrate, globin. The region near the NH₂-terminal activation peptide does not cover the active site in the intermediate δ but subsequently binds to this active site when
the intermediate $\theta$ is formed. We have previously argued (2) that the pepsin active site is responsible for the hydrolysis of intramolecular activation. The results of this paper are consistent with that idea.$^2$

Obviously, the above arguments depend on the assumption that both pepstatin and globin bind to the pepsin active site in the pepsinogen intermediate $\delta$ and not to a different locale on the enzyme surface. It is therefore important to examine the evidence for this assumption. (a) In a separate work (5) we have shown that fragments of pepstatin are competitive inhibitors of pepsin and the pepsin is probably a transition-state analog of pepsin. (b) Low resolution x-ray crystallographic studies by Subramanian and co-workers (16) on the acid protease from Rhicopus chimeris, an enzyme with amino acid sequence homology to pepsin (17), indicates that pepstatin binds in the cleft of the enzyme and is in contact with the binding locale of the active-site directed reagent 1,2-epoxy-3-$(p$-nitrophenoxy)propane (18). (This reagent covalently modifies Asp-32 in the pepsin molecule (15).) (c) The binding of globin to pepsinogen intermediate $\delta$ inhibits "competitively" the intramolecular activation (Fig. 5 and arguments under "Results"). As a consequence, most probably, the binding is to the same site. All three of the above pieces of evidence suggest strongly that the pepsin active site is exposed and capable of binding pepstatin inhibitors or substrates in the intermediate $\delta$ and involved in the intramolecular scission of the peptide bond to form pepsin and the activation from the intermediate $\theta$. Even so, they do not eliminate entirely the possibility that the intramolecular activation is carried out by a separate active site which is impaired by the inhibitors either due to the juxtaposition of the two sites or due to conformational change upon inhibitor binding. However, we have no supporting evidence for these alternatives and, therefore, would suggest that the schemes shown in Figs. 7 and 8 represent the most likely events.

In Figs. 7 and 8, the intermediate $\delta$ is placed in the obligatory pathway of the intramolecular activation of pepsinogen. This scheme is consistent with all the results reported in this paper. However, it should be pointed out that current kinetic data cannot exclude the scheme in which intermediate $\delta$ is non-obligatory (Scheme 2, Fig. 1).

Recently, Dykes and Kay (19) reported preliminary findings on the isolation of an activation peptide which resulted from the activation of pepsinogen in the presence of pepstatin. These results suggested that the first cleavage site of pepsino-

\[ \text{PEPSINOGEN} \xrightarrow{H^+} \text{INTERMEDIATE} \xrightarrow{\theta} \text{INTERMEDIATE} \xrightarrow{\phi} \text{INTERMEDIATE} \xrightarrow{\alpha} \text{PEPSIN} + \text{PEPTIDE} \]

**Fig. 8.** Mechanism of pepsinogen activation. The large circle is the main protein molecule; the small square and circle are, respectively, undeveloped and developed active sites which each contain two carboxyl groups; the line represents the activation peptide.

$^1$ In a 2.7-Å crystal structure of pepstatin recently revealed by Andreeva (14), the NH$_2$ terminus of the enzyme is located in the vicinity of the substrate binding cleft and catalytic aspartyl residues 32 and 215 (15). This is consistent with the involvement of the pepsin active center in the intramolecular pepsinogen activation.
been found, the zymogen of an acid protease from human seminal plasma has been purified recently (26). The properties of this zymogen appear to be very similar to those of pepsinogen, and thus it may be activated by means of a similar mechanism.

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