Kinetics and Mechanism of Pepsinogen Activation*

(Received for publication, March 3, 1972)

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SUMMARY

The spontaneous and pepsin-catalyzed activation of pepsinogen has been observed and analyzed kinetically. At appropriate protein concentrations (1 mg per ml or less), a kinetically first order reaction was observed in the pH range 1 to 3, implying an intramolecular activation mechanism. Substitution of the first order reaction came from a linear plot of log pepsinogen concentration versus time, with an ordinate intercept at the log of the initial pepsinogen concentration. Moreover a 10-fold dilution in protein concentration did not diminish the half-life of the activation reaction. The first order rate constant of 4.7 per min at 28°C was comparable to the $k_{cat}$ values observed for peptic hydrolysis of synthetic peptide substrates.

At pH 4, we observed kinetics consistent with a mixed reaction mechanism involving the bimolecular reaction of a pepsin molecule and a pepsinogen molecule as well as an intramolecular activation reaction. Below pH 3, this second order process was studied by observing the initial rate of pepsinogen activation in the presence of pepsin. When the pH profiles of the first and second order rate constants were compared, the first order rate constant declined much more rapidly as pH increased than did the second order rate constant. This fact explains the change from a predominantly intermolecular activation mechanism at pH 4 to an intramolecular activation below pH 3.

* This work was supported by Research Grants AM-01107 and AM-06487 from the National Institutes of Health. A preliminary report of this work has been presented (1).
‡ Recipient of Career Development Award 1-K04-GM-21,736.
§ Recipient of Career Development Award 5-K01-AM-38,638.
EXPERIMENTAL PROCEDURE

Materials

The porcine pepsinogen used in these investigations was purified from a crude commercial preparation (Nutritional Biochemical Corporation) by means of chromatography using Sephadex G-200 and DEAE-cellulose columns. A few control experiments were performed using Worthington crystalline pepsin, PGC. Crystalline pepsin was the Worthington preparation, PM.

Hemoglobin used in the determination of proteolytic activity was obtained from Pentex and was purified further by dialyzing against three changes of distilled water. The hemoglobin was preserved as a lyophilized powder. A fresh hemoglobin solution was made up against water adjusted to pH 9 with ammonia and then against three changes of distilled water. The hemoglobin was preserved as a lyophilized powder. A fresh hemoglobin solution (20 mg per ml) in 0.1 M sodium citrate buffer, pH 7.5, was prepared each day. All other chemicals were of the highest purity available from commercial sources and were used without further purification.

For the activation experiments, solutions of different pH values were used, as follows: pH 1.0, 0.1 M KCl; pH 1.4, 0.1 M KCl; and pH 2, 0.1 M sodium citrate. All three were adjusted to the correct pH with HCl. Buffers at pH 3.0 and 4.0 were prepared by mixing appropriate amounts of 0.1 M sodium citrate and 0.1 M citric acid.

Methods

Pepsinogen Purification—Crude pepsinogen was dissolved in 0.02 M Tris-chloride buffer, pH 7.5. This solution was placed on a pre-equilibrated Sephadex G-200 column. Elution was carried out with the same Tris buffer. Those fractions showing a high potential specific activity of pepsin were pooled.

The pooled fractions were then rechromatographed on a DEAE-cellulose column using a linear salt gradient moving from 0.02 M Tris-chloride, pH 7.5, to 0.2 M sodium chloride and 0.02 M Tris-chloride, pH 7.5. Fractions constituting a peak of uniform specific activity, whose maximum concentration was at 0.27 M NaCl, were pooled, lyophilized, and used as the starting material in subsequent experiments. The zymogen prepared in this manner had the same specific activity as the Worthington preparation PGC and a specific activity 15% higher than that of the commercial crystalline pepsin.

Activation of Pepsinogen—The general scheme for these experiments was this: The pH of a suitable pepsinogen solution was rapidly lowered to an activating condition. After an appropriate interval, the activating mixture was diluted as the pH was raised to 8.5. This stopped activation and irreversibly denatured any pepsin that had been formed. The remaining pepsinogen was then activated and assayed.

Conditions and procedures for a typical experiment at a pepsinogen concentration of 1 mg per ml, pH 2.0, were as follows. Fifteen milligrams of pepsinogen were dissolved in 7.5 ml of water. Then, 100 µl of this solution were stirred magnetically in a test tube immersed in a 28° thermostated vessel. One hundred microliters of pH 2.0 buffer were added to the pepsinogen solution, and, after the appropriate time interval, which was measured with a stopwatch, 300 µl of 0.5 M Tris-chloride, pH 8.5, were added. These additions, made with a syringe, were as instantaneous as possible. A reservoir of pH 2.0 buffer was kept in the thermostated bath and the solution was drawn into the syringe just before use. The process was repeated for each predetermined time interval (i.e. 1, 2, 4, etc., sec). After at least 20 min of standing in alkaline solution, 100 µl of each solution were assayed for remaining pepsinogen activity. Other activating pepsinogen concentrations were attained by appropriate preparation of the initial solution. Other activating pH values were attained using solutions of different pH values in the first syringe. When activation was carried out in the presence of pepsin, the buffer added in the first syringe contained an appropriate amount of pepsin.

Assay—The potential proteolytic activity was assayed at 28° using hemoglobin as a substrate. A 100-µl aliquot of pepsinogen was added to 1 ml of 0.2 M sodium citrate buffer, pH 2.0, and allowed to activate for 15 to 20 min. A 1-ml hemoglobin solution was added to each tube; then, after exactly 10 min, 1 ml of 10% trichloroacetic acid solution was added. The mixture was filtered on a Whatman No. 50 filter paper and the optical density of the filtrate was read at 280 nm against a blank containing no enzyme. In the case of very dilute pepsinogen in the activating solution (e.g. 0.1 mg per ml), the assay time was lengthened to 30 min and the assay temperature was raised to 35°. All assay conditions used produced optical densities at 980 nm of 1.2 or less. Control experiments showed that in this enzyme concentration range the optical density change is directly proportional to the pepsin concentration.

Control Experiments—In order to ascertain appropriate time intervals for denaturation of pepsin at pH 8.5 and complete activation of the remaining pepsinogen for assay, we performed two experiments. First, a 1 mg per ml pepsinogen solution was activated at pH 2.0 for 45 sec and allowed to remain at pH 8.5 for times varying from 1 to 60 min; then the remaining pepsinogen was activated at pH 2.0 for 20 min and assayed. The pepsin concentration at assay declined to a constant value within 12 min and remained so when exposure to pH 8.5 was continued for up to 60 min. Therefore, at least 12 min were required for complete pepsin inactivation under these conditions. Second, when another 1 mg per ml pepsinogen solution was activated for 45 sec, inactivated for 30 min, and then activated at pH 2.0 for time intervals varying from 0 to 60 min, the pepsin assay value rose to a constant value in 8 min. Significantly, the constant value failed to rise even after 60 min of incubation at pH 2; therefore, reassembly of denatured pepsin did not occur to a measurable extent.

Mathematical and Statistical Treatment of Data—The first order kinetic data was fitted to the equation

\[ \ln [Pgn] = -kt + \ln [Pgn]_0 \]  

(2)

where [Pgn] is the pepsinogen concentration at time \( t \), \([Pgn]_0\) is the initial pepsinogen concentration, and \( k_t \) is the first order rate constant. The best value of \( k_t \) and a calculated \([Pgn]_0\) were determined from a weighted least squares fit of the observed sets of \([Pgn]\) and \( t \) (17). The error used to determine the weight was

\[ e = 0.015 + 0.08 [Pgn] \]  

(3)

where \( e \) is the error, 0.015 is the experimentally determined error in the assay blank, and 0.08 is our estimate of the fractional experimental error because of pipetting, etc.; \( e \) squared is inversely proportional to the weight.

The second order constants were determined using the rate
In this case, the rate equation was Equation 4, and its integrated form was

\[
-\frac{d[Pgn]}{dt} = k_1[Pgn] + k_2[Pgn][Pep]
\]  

where \(-\frac{d[Pgn]}{dt}\) is the rate of disappearance of pepsinogen, \(k_1\) is the second order rate constant, \([Pep]\) is the pepsin concentration, and \(k_2\) and \([Pgn]\) have the same definition as in Equation 4. An initial rate of pepsinogen disappearance is calculated from a least squares determination of the slope of the linear portion of that plot of \([Pgn]\) versus \(t\) when pepsin is added to the activation mixture. Then, using an average \(k_1\) value which was previously determined and initial concentrations of zymogen and enzyme, Equation 4 was solved for \(k_2\).

At pH 4.0, a mixed reaction mechanism which incorporates both first order and autocatalytic pathways was assumed. In this case, the rate equation was Equation 4, and its integrated form was

\[
[Pgn] = \frac{[Pgn_0](k_1 + k_2[Pgn_0])e^{-(k_1+k_2[Pgn_0])t}}{k_1 + k_2[Pgn_0]e^{-(k_1+k_2[Pgn_0])t}}
\]  

where \([Pgn_0]\) is the initial pepsinogen concentration and all other variables have the same definition as in Equation 4. Equation 5 assumes a negligible initial pepsin concentration. A trial-and-error curve-fitting procedure was used to deduce the correct values for the rate constants \(k_1\) and \(k_2\). An Olivetti Programma 101 calculator was used for all calculations.

**RESULTS**

**Proof of Existence of Intramolecular Activation**—We recorded data for the activation of pepsinogen at pH 1.0, 1.4, 2.0, and 3.0 and at protein concentrations of 0.1, 0.5, and 1.0 mg per ml. Semilogarithmic plots of the pepsinogen concentrations versus time appeared linear even when followed to a pepsinogen concentration one-third of the initial value. Sample plots are shown in Fig. 1. The data therefore fitted the first order kinetic equation (Equation 2 above). The observed and calculated initial concentrations agreed closely. Of the 68 experiments performed, 63 showed less than a 4% difference between observed and calculated initial concentrations. The maximum difference was 7%. Moreover, as shown in Table I, the first order rate constant \(k_1\) did not change significantly when the pepsinogen concentration was varied by a factor of 10. Therefore, because the semilogarithmic plot was linear and had a proper intercept and because the activation rate constant was not concentration dependent, we concluded that first order, intramolecular activation of pepsinogen was taking place.

**TABLE I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Pepsinogen concentration</th>
<th>pH</th>
<th>(k_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>1.0</td>
<td>4.0</td>
<td>0.008 ± 0.0005 (2)</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>4.0</td>
<td>0.008 (1)</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>3.0</td>
<td>0.39 ± 0.05 (7)</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>3.0</td>
<td>0.53 (1)</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>2.0</td>
<td>2.6 ± 0.2 (22)</td>
</tr>
<tr>
<td>O</td>
<td>1.0</td>
<td>2.0</td>
<td>2.6 ± 0.1 (5)</td>
</tr>
<tr>
<td>W</td>
<td>1.0</td>
<td>2.0</td>
<td>2.4 ± 0.1 (3)</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>2.0</td>
<td>2.9 ± 0.4 (5)</td>
</tr>
<tr>
<td>D</td>
<td>0.1</td>
<td>2.0</td>
<td>2.7 ± 0.2 (3)</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>1.4</td>
<td>3.9 ± 0.3 (10)</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>1.4</td>
<td>4.6 ± 0.1 (2)</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>1.0</td>
<td>4.1 ± 0.7 (9)</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>1.0</td>
<td>3.8 (1)</td>
</tr>
</tbody>
</table>

* Key for the various pepsinogen preparations: D = our preparation which was used in most experiments; O = a collection of three other preparations; W = Worthington PGC pepsinogen.

b First order rate constant.

c The form of the data is arithmetic mean ± root mean square deviation (number of observations).

The pH 3.0 activation experiments were followed just 60 sec, even though only about one-third of the pepsinogen had been activated by this time. The latter part of the reaction would probably have shown autocatalytic activation in addition to the first order reaction (see pH 4.0 results, below).

At high protein concentrations (5 to 15 mg per ml) slow activation was observed, probably because of zymogen-zymogen interactions. Centrifugation (18) and nuclear magnetic resonance experiments have confirmed the existence of such interactions.

**pH 4.0 Pepsinogen Activation**—The plot of pepsinogen concentration versus time for the pH 4.0 activation experiment (Fig. 2) did not decline exponentially, as did similar plots from experiments at lower pH. Rather, an initial lag period was followed by a more rapid decline in zymogen concentration. Note, moreover, that the half-life for this reaction was about 16 min, compared with 1.77 min at pH 3.0 and 0.266 min at pH 2.0. The pH 4.0 activation reaction was predominantly autocatalytic.

1 M. Hunkapiller, personal communication, 1971.
(2), while at pH 2.0, with similar protein concentrations, only first order activation could be detected (i.e. intramolecular activation). Therefore, we assumed that the reaction mechanism at pH 4.0 might be characterized by a significant contribution from each of these pathways. Consequently, we attempted to fit the concentration versus time curve using the integrated form (Equation 5) of the rate equation for the mixed mechanism (Equation 4). A typical set of raw data and calculated curve are shown in Fig. 2. The average values of the rate constants $k_1$ and $k_2$ are included in Tables I and II.

**Determination of Autocatalytic Rate Constant $k_2$.** Below pH 3.0, the rate of pepsinogen activation in the presence of added pepsin was used to determine the rate constant $k_2$. An initial rate of activation determines $k_2$ if $k_1$ is known. The equations for this treatment appear in "Methods." In all experiments, a pepsinogen concentration of 1 mg per ml was used. Pepsin concentrations were 1, 2, or 5 mg per ml. The resulting rate constants are shown in Table II. The root mean square deviations were larger than in the $k_1$ determinations, but we could detect no systematic error. For example, the value of $k_2$ varied randomly as added pepsin concentration was changed.

**Dependence of Rate Constants on pH—**The rate constant for the intramolecular activation, $k_1$, rose rapidly from pH 4.0 to 1.4, but then did not change significantly between pH 1.4 and 1.0. If the pH-rate profile for $k_1$ is interpreted to show that the intramolecular reaction requires the protonation of a single group of $pK_a$ 2.1, the pH-independent value of $k_1$ which can be calculated is 4.67 per min. The autocatalytic rate constant, $k_2$, rose from pH 4.0 to a maximum at pH 3.0. The decline was less rapid below pH 3.0 than above this pH. Fig. 3 shows the dependence of both rate constants on pH. The decline in $k_1$ between pH 2.0 and 4.0 was 44 times greater than the decline in $k_2$ over the same pH range.

**DISCUSSION**

Our experiments indicate that pepsinogen is capable of either intramolecular or autocatalytic activation. The relative importance of these pathways depends on several factors. The presence of added pepsin accentuates the autocatalytic reaction. High zymogen concentration favors the second order, autocatalytic pathway, while low pH, below 3.0, produces dominance by the intramolecular reaction. Strictly speaking, our studies do not eliminate the possibility that pepsinogen can be activated by the action of another zymogen molecule, as has been observed for prorennin and trypsinogen (4, 12). However, this mechanism need not be invoked to explain our observed results. So, if a zymogen-zymogen activation does occur its rate constant must be quite small. Although the peptide cleaved from pepsinogen, the so-called pepsin inhibitor, has been shown to have affinity for pepsin (19), the pepsin inhibitor should affect neither $k_1$, which is determined from an intramolecular process, nor $k_2$, which is determined from an initial rate study.

The active center of pepsin has been shown to contain two aspartic acid residues. The first, which is a part of the sequence Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu (20, 21), can be modified by diazo compounds (22–24) and has a $pK_a$ of about 5.0 (25). The second, which is contained in the sequence Ile-Val-Asp-Thr-Gly-Ser-Ser-Asn (26), reacts with certain epoxide compounds (27) and has a $pK_a$ below 3.0 (28). Two recent, independent suggestions for the catalytic mechanism of pepsin incorporate proton donation from the aspartic acid which can be modified by diazo compounds and nucleophilic attack by the epoxide-sensitive carboxylate ion (28, 29). For several reasons, we believe that unimolecular pepsinogen activation probably involves the same active center and catalytic residues that are used in the peptic hydrolysis of peptide substrates. Since pepsinogen reacts with neither diazo compounds (23) nor specific epoxide modifiers (27), its catalytic aspartic acid residues may be covered by the NH2-terminal chain which will be removed.

**Table II**

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_2$ $^{a}$ (min$^{-1}$ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>0.20 ± 0.03 (3)</td>
</tr>
<tr>
<td>3.0</td>
<td>2.6 ± 0.6 (6)</td>
</tr>
<tr>
<td>2.0</td>
<td>1.3 ± 0.4 (6)</td>
</tr>
<tr>
<td>1.4</td>
<td>1.5 ± 0.8 (6)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0 ± 0.2 (9)</td>
</tr>
</tbody>
</table>

$^{a}$ Second order autocatalytic rate constant.

$^{b}$ The form of the data is: arithmetic mean ± root mean square deviation (number of observations).

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**Fig. 3.** pH dependence of the first and second order rate constants, $k_1$ and $k_2$. The units for $k_1$ are (min$^{-1}$); for $k_2$ are (min × mg per ml)$^{-1}$. The vertical lines indicate root mean square deviations in the rate constant determinations.

**Fig. 4.** Schematic representation of the intramolecular activation of pepsinogen. The catalytic aspartic acid residues of pepsin are described under "Discussion."
upon activation. The residues leucine and isoleucine (6, 15), which are present on either side of the bond cleaved during activation, would fit well into the hydrophobic binding sites near the active center (29, 30). The $k_1$ pH rate profile which we determine resembles some of the $k_{cat}$ and $k_{cat}/K_m$ profiles for peptide hydrolysis by pepsin (30). The $k_1$ value of 2.6 per min at pH 2 for the activation reaction is comparable with $k_{cat}$ values at the same pH for pepsin hydrolysis of synthetic substrates; those values range from 0.1 to 46 per min. Consequently, the intramolecular cleavage of pepsinogen probably takes place at the same active site that is used by the resulting enzyme pepsin. Even though the catalytic machinery of pepsin may remain intact in the zymogen, some postactivation conformational change would be necessary to remove the new NH$_2$-terminal region of the peptide chain from the active center so that it does not prohibit subsequent substrate binding. All of these ideas are summarized in Fig. 4, which portrays the intramolecular activation reaction schematically.

The pH dependence of our intramolecular and autocatalytic activation rate constants explains the change in predominant activation mechanism when pH is lowered. Calculations based on the mixed first and second order mechanism show that, for our rate constants at pH 2.0, 83% of a completely activated pepsinogen solution (1 mg per ml) would have followed the first order path-way, while at pH 4.0 only 16% would come from the intramolecular reaction. The upper limb of the $k_1$ rate profile is consistent with the pH$_a$ of about 4 which has been observed for the diazo-sensitive aspartic acid residue in pepsin (24). Assuming that intramolecular activation takes place with the same mechanism as pepsin catalysis, the $k_1$ rate profile indicates that the pH$_a$ of the diazo-sensitive residue is probably lower in the zymogen than in the active enzyme. In conclusion, the activation of pepsinogen is a complicated reaction which can be influenced by several factors. We offer kinetic evidence for the existence of two activation pathways, intramolecular and autocatalytic. They both exist over the entire pH range of 1.0 to 4.6, but their relative importance changes. Two reasons emerge, then, to explain why the activating conditions suggested by Rajagopal et al. (16) yield homogeneous pepsin. First, at pH 2 the reaction is rapid and autodigestion does not occur during the activating time. Second, the intramolecular reaction which predominates at pH 2.0 produces cleavage at a single point, whereas autocatalytic activation could generate several NH$_2$-terminal.

Acknowledgments—The authors wish to thank Dr. David Huang for purifying some of the pepsinogen and Mr. Mike Hunkapiller for making his results and conclusions available to us prior to publication. Ms. Barbara Cox provided valuable assistance in preparing the paper.

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