Intramolecular Activation of Porcine Pepsinogen*

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SUMMARY

Conversion of pepsinogen to pepsin at acid pH involves an intramolecular reaction in which the unproteolyzed zymogen cleaves itself. This conclusion is based upon experiments in which pepsinogen, at low concentrations, was activated in the presence of substrate, hemoglobin. Under these conditions, the activation of pepsinogen is independent of pepsinogen concentration, and addition of pepsin does not enhance the rate of autoactivation.

The activity of Sepharose-bound pepsinogen was independent of the average amount of pepsinogen bound per g of Sepharose, suggesting that the activation process is not caused by neighboring molecules mutually activating. Apparently, in more concentrated solutions that were previously employed for autoactivation, the concentration of pepsinogen was such that the reaction could be catalyzed by one of the products (an autocatalytic reaction). When the activation process is performed at low pepsinogen concentration in the presence of substrate, or in columns of Sepharose-bound pepsinogen, the results indicate that reaction between a trace of pepsin and pepsinogen or between molecules of pepsinogen is not a prerequisite for activation.

Amino-terminal studies on Sepharose pepsinogen yielded leucine as the amino-terminal acid. Exposure of the Sepharose pepsinogen to acid yielded leucine and isoleucine as amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid failed to yield isoleucine as the amino-terminal amino acids. Treatment of base-donated pepsinogen with acid fa...
first converted to the ferric state with potassium ferricyanide, according to the method of Austin and Drabkin (13). After extraction of the heme with methyl ethyl ketone, the globin was exhaustively dialyzed against water. The precipitate which formed at pH 7 was separated by centrifugation and then dissolved in 0.1 N HCl. The absorbance at 280 μm of the globin solution was 20. All spectrophotometric measurements were performed in cuvettes of 1-cm path length.

Coupling of Protein to Sepharose—Pepsinogen or pepsin was coupled to cyanogen bromide activated Sepharose beads by a slight modification (14) of the method of Porath, Axen, and Ernæn (15). Sepharose 4B was washed with distilled water on sintered glass funnels until the absorbance at 280 μm of the filtrate was below 0.02. Five grams of the moist Sepharose (partially dried on the funnels) were suspended in 10 ml of water and the cyanogen bromide was initially dissolved in 5 ml of water. The suspension was washed, activated Sepharose was added to protein solutions of various concentrations. The protein concentration varied from 1 to 7 mg per ml and the buffer was either 0.1 M sodium phosphate, pH 6.7, or 0.1 M sodium bicarbonate, pH 8.8. The protein-Sepharose suspensions were shaken overnight at 4°C and then washed (sintered glass funnel) with 0.1 M sodium bicarbonate until the absorbance at 280 μm was below 0.02. The amount of protein bound was estimated from the absorbance at 280 μm recovered in the filtrate. The exact amount bound was determined by amino acid analyses (16).

Denaturation of Pepsinogen—This was accomplished by incubation at pH 11.5 at 29°C for 2 hours (6, 17).

Assay Procedures—Proteolytic activity in solution was measured with the hemoglobin assay developed by Anson (18), with dialyzed hemoglobin solutions as substrate (17). The reaction was terminated by the addition of equal volume of 10% trichloracetic acid. The mixture was allowed to stand at room temperature for 10 to 30 min, centrifuged, and the absorbance at 280 μm of the supernatant measured.

Preliminary Digestion of Hemoglobin—To a 2.1% solution of hemoglobin at pH 1.8 and 37°C pepsinogen was added to a final concentration of 1 μg per ml. The digests were incubated at 37°C for 10 min. The peptic activity was destroyed by bringing the digest to pH 11.5 and keeping it at this pH and 23°C for 2 hours. Prior to the addition of fresh pepsinogen the hemoglobin digest was acidified to pH 1 (to dissolve the heme and the globin) and the pH of the digest was adjusted to the desired value with 1 N NaOH.

Activity Measurements on Sepharose-bound Pepsinogen—Pepsinogen Sepharose was diluted to the desired concentration of pepsinogen by adding the appropriate amounts of unactivated, washed Sepharose. The mixture of pepsinogen Sepharose and Sepharose was then added to columns (8.5 × 0.5 cm). The total amount of Sepharose added to each column was approximately 0.6 g. The total amount of pepsinogen per column varied from 5 to 100 μg. To measure the rate of globin digestion, 0.5 ml of globin solution was added to the column at room temperature (20-21°C). After all the globin was adsorbed on the Sepharose, 0.01 N HCl was added to wash off the globin. The time between the adsorption of the 1st drop of the slightly colored globin solution onto the Sepharose and the emergence of 1.5 ml was recorded. To compare the digestion of globin by Sepharose-bound pepsinogen and activated pepsinogen in solution, the time between the adsorption of the 1st drop of globin onto the Sepharose and the emergence of the 1st slightly colored drop was considered to be the average time that the globin was in contact with the pepsinogen Sepharose. The amount of digestion was measured by the appearance of trichloracetic acid-soluble peptides.

To check whether any activity was eluted from the column at pH 2, columns containing 1.5 to 2.0 mg of Sepharose-bound pepsinogen were prepared. The columns were first washed with 0.02 M sodium phosphate, pH 7.4, 0.15 M NaCl, and then with water until the absorbance of the eluate at 230 μm was less than 0.02. The columns were brought to acid pH with 0.015 N HCl and the eluate was collected. Activity measurements on the eluate were done with the hemoglobin assay.

Immunization Procedure—Rabbits were immunized by an injection of 4 mg of either pepsin or pepsinogen in complete Freund's adjuvant at multiple intradermal sites. Ten days after the injection a booster of 2 mg of protein in complete Freund's adjuvant was given (1 mg was injected at multiple intradermal sites and 1 mg was injected intramuscularly). Starting 8 days after the booster, blood was collected weekly from the marginal ear vein. After the antibody titer of the various individual sera was checked by the precipitin test, the anti- pepsin sera and the antipepsinogen sera were separately pooled.

Quantitative Precipitin Test—Precipitin reactions were performed with whole antisera. Increasing amounts of the antigen were added to a constant amount of the antibody. The precipitates formed after 1 hour at 37°C and overnight at 4°C were washed, dissolved in 0.1 N NaOH, and quantitatively determined by measuring the absorbance at 280 μm within 10 min.

Amino Acid Analyses and Determination of Amino-terminal Amino Acids—Acid hydrolysis of proteins and Sepharose-coupled protein and determination of amino acid composition was carried out as described by Moore and Stein (16). The 1-dimethylaminonaphthalene-5-sulfonyl method of Gray (19) was slightly modified (20). The dansyl derivatives of the amino acids were extracted with an acetonitrile-acid mixture (3:2) and identified by thin layer chromatography (21) as described previously (20). Control experiments with Sepharose-bound alanine were performed.

RESULTS

Activation of Pepsinogen in Presence of Substrate—Herriott (5) has shown that the conversion of pepsinogen to pepsin is accelerated by addition of enzyme and that the process is dependent on the pepsinogen concentration (5). We have undertaken a study of the activation of pepsinogen in the presence of hemoglobin at low (0.5 to 5 μg per ml) concentrations of pepsinogen. In initial tests, in which pepsinogen was added to hemoglobin solutions (at pH 1.8), an increase in pepsinogen concentration resulted in an apparent increase in the observed specific activity as measured by the appearance of trichloracetic acid-soluble peptides.

1 The abbreviation used is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl-.
peptides. There was a lag period in the appearance of these peptides and the plot of activity versus time was concave. These results could be caused by activation of pepsinogen by traces of pepsin and subsequent interaction between catalytically active molecules. It is equally probable, however, that the dependence of the rate of hemoglobin digestion on the concentration of pepsinogen resulted from a change in the susceptibility of the substrate (hemoglobin) to digestion during the course of the reaction. Indeed, when the hemoglobin was initially digested (see "Experimental Procedure") prior to the addition of pepsinogen, the plot of activity versus time was linear and the observed specific activity was independent of pepsinogen concentration, as shown in Fig. 1.

In a further test hemoglobin was digested by a mixture containing 90% pepsinogen and 10% pepsin (the zymogen and the enzyme were separately added to the substrate). The results suggested that at pH 1.8 pepsin did not enhance the conversion of pepsinogen to pepsin. Actually, it can be seen from Fig. 2 that under these conditions the proteolytic activity is identical, irrespective of whether pepsinogen or pepsin was added to the hemoglobin.

It is known, however, that at pH 1.8 the conversion of pepsinogen to pepsin is very rapid and while the experiments described above minimize the possibility that the proteolytic activity observed is a result of activation of pepsinogen molecules by traces of pepsin, it is still possible that the activation process is so fast that it could not be detected.

Therefore, we performed similar experiments under conditions where the course of hemoglobin digestion depends on whether pepsinogen or pepsin is added. The results of such experiments are presented in Fig. 3. When pepsinogen is added to hemoglobin solutions at pH 3 and 23°C there is a distinct lag period in the appearance of proteolytic activity. This lag period could represent the removal of the pepsin inhibitor peptide (5-7) from pepsin. At pH 3, the amount of trichloracetic acid-soluble peptides resulting from 20-min digestion of hemoglobin by pepsin was approximately 4 times the amount obtained when pepsinogen
was added to the same amount of substrate. When hemoglobin was incubated with a mixture of pepsin and pepsinogen (separately added to the substrate), the rate of digestion equaled the sum of the rates observed when hemoglobin was incubated with each component separately. It can be seen that the rate of hemoglobin digestion by the mixture is significantly lower than the rate obtained by an equivalent amount of pepsin. At pH 3 and 23°C, just as is found at pH 1.8 and 37°C, the specific activity is independent of pepsinogen concentration. These results support the notion that the activity displayed by the pepsinogen solution did not result from activation by pepsin or by the interaction of several molecules. Apparently, when activation is performed at low pepsinogen concentration in the presence of hemoglobin, the substrate may act as a competitive inhibitor and the activation process ceases to be autocatalytic.

Activity of Sepharose-bound Pepsinogen—In order to study the activation under conditions in which interaction between pepsinogen molecules is minimized, we have immobilized the pepsinogen by covalently binding it to Sepharose. It can be seen from Table I that the amount of pepsinogen that bound to Sepharose was dependent on the concentration of pepsinogen, the concentration of Sepharose, and the mode of cyanogen bromide addition to the reaction mixture. For example, when 2 g of Sepharose were added to 1 ml of a solution of 1.0 mg per ml of pepsinogen, 85% of the pepsinogen added bound to Sepharose. In contrast, when the concentration of Sepharose was 0.25 g per ml only 21% of the pepsinogen added bound to Sepharose. When dissolved cyanogen bromide was used, the percentage of pepsinogen that bound to Sepharose was significantly higher than when solid cyanogen bromide was used. The amino acid composition of the Sepharose-pepsinogen preparations indicated that the entire molecule was bound to the resin.

The activation process was studied only with preparations from which no detectable pepsin activity was eluted upon exposure of Sepharose-bound pepsinogen at pH 2. At pH 6.7 pepsinogen was bound to Sepharose through relatively few bonds (29) and upon exposure of this preparation to pH 2, 50% of the peptic activity bound to Sepharose was released. When the zymogen was bound to Sepharose at pH 8.8, the amount of activity eluted was dependent upon the amount of pepsinogen bound to Sepharose. Detectable activity was eluted when more than 2.5 mg of pepsinogen was bound to 1 g of Sepharose. When there were less than 2.5 mg of pepsinogen per g of Sepharose, no detectable activity was eluted.

The amino acid composition of these Sepharose-pepsinogen preparations was not altered by exposure to pH 2. Apparently all the peptide fragments resulting from exposure of the Sepharose pepsinogen to acid are covalently bound to the Sepharose.

The Sepharose-bound pepsinogen was poured into columns and the proteolytic activity of the columns was measured with globin (hemoglobin was not suitable for this purpose because the home precipitated on top of the column). The proteolytic activity of Sepharose-bound pepsinogen columns was about 30% of the activity of pepsinogen in solution. As shown in Fig. 4, the amount of globin digested was proportional to the amount of pepsinogen in a column. The specific activity of Sepharose-bound pepsinogen preparations in which the amount of pepsinogen was less than 2.5 mg per g of Sepharose was independent of the ratio of pepsinogen to Sepharose. For example, preparations originally containing 2 and 0.45 mg of pepsinogen per g of Sepharose were mixed with unactivated Sepharose to give 10 μg of pepsinogen per column. The specific activity (absorbance at 280 μm x min⁻¹ x mg⁻¹) of the pepsinogen in these columns was 2.6 and 2.3, respectively. Apparently the activation process is independent of the average distance between pepsinogen molecules and interaction between neighboring molecules can probably be excluded as a major factor in the activation process.

It has been previously shown (6, 17) that acid-activated pepsinogen is irreversibly inactivated by high pH. Similarly, exposure of acid-activated Sepharose-bound pepsinogen to pH 8.5 and 23°C for 1 hour brought about an irreversible loss of proteolytic activity.

Amino-terminal Studies—To see whether the proteolytic activity of Sepharose-bound pepsinogen is caused by unprotected or cleaved pepsinogen we determined the amino-terminal prior and subsequent to exposure of Sepharose-bound pepsinogen to acid. Prior to acid exposure, the only spot observed was that corresponding to dansyl-leucine; after acid exposure, both dansyl-leucine and dansyl-isoleucine were detected. Dansylation of base-denatured pepsinogen which was exposed to pH 2 yielded only leucine as the amino-terminal amino acid. These experiments suggest that cleavage of the peptide bond which results in the exposure of isoleucine involves an enzymatic process.
Fig. 5. Precipitin curves of pepsin and pepsinogen with homologous and heterologous antisera. Either pepsinogen (●) or pepsin (△) was added to 0.4 ml of various sera. A, antipepsinogen passed through Sepharose-bound pepsinogen; B, antipepsinogen passed through Sepharose-bound pepsinogen column which had been brought to pH 2 for 30 min and subsequently to pH 7.4; C, antipepsinogen passed through Sepharose-bound pepsin; D, antipepsin sera; E, antipepsin sera. Antipepsinogen sera (5.8 ml) was passed through columns containing 150 μg of antigen (for A through C).

Immunological Studies—We were also interested to see whether the cleavage of the peptide bond (possibly the glutamyl-isoleucine bond (9)) in Sepharose-bound pepsinogen gave rise to a marked conformational change in the protein. From Fig. 5, D and E it can be seen that a large amount of highly cross-reacting antibodies were obtained when rabbits were intradermally injected with pepsin or pepsinogen. To find out whether Sepharose-bound pepsinogen removes preferentially antipepsinogen or antipepsin antibodies, sera were passed through columns containing limiting amounts of pepsinogen. When 5.8 ml of antipepsinogen was passed through a column containing 150 μg of pepsinogen, 21% of the antipepsin and 50% of the antipepsinogen activity were removed (see Fig. 5A). In contrast to the pepsinogen columns which preferentially bound antipepsinogen activity, Sepharose-pepsin columns did not show preference for either the antipepsin or antipepsinogen activities of the antipepsinogen sera (Fig. 5C). Exposure of the Sepharose-pepsinogen column to pH 2 did not alter the amount of antipepsinogen and antipepsin activity which bound at pH 7.4 (compare Fig. 6, A with D). From these experiments it was concluded that there was no detectable loss of pepsinogen-specific antigenic determinants, nor appearance of new pepsin-specific antigenic determinants upon activation of the Sepharose-bound pepsinogen.

Discussion

We conclude from the experiments presented in the previous sections that the conversion of pepsinogen to pepsin at acid pH involves an intramolecular reaction in which the unproteolyzed zymogen cleaves itself. Kinetic studies of the activation of pepsinogen in the presence of the substrate, hemoglobin, showed that the rate of activation was independent of pepsinogen concentration and was not enhanced by the addition of pepsin.

These results, together with the finding that the activity of Sepharose-bound pepsinogen was independent of the average amount of pepsinogen bound per g of Sepharose (i.e. the average distance between adjacent molecules), showed that a bimolecular reaction between traces of pepsin and pepsinogen or between molecules of pepsinogen is not a necessary step in the activation process of pepsinogen. Apparently, in more concentrated solutions that were previously employed for autoactivation, the concentration of pepsinogen was such that the reaction could be catalyzed by one of the products, i.e. the reaction was autocatalytic (5). When the activation process is performed at low pepsinogen concentrations in the presence of hemoglobin, the substrate may act as a competitive inhibitor and the reaction ceases to be autocatalytic.

The appearance of the pepsin amino-terminal acid (isoleucine, see Reference 9) upon exposure of Sepharose-bound pepsinogen to acid suggests that the unproteolyzed pepsinogen molecule may be capable of splitting peptide bonds. At least, it can cleave itself in an intramolecular reaction. Indeed, this cleavage appears to be enzymatic, requiring a particular alignment of catalytic residues, as indicated by our findings that acid exposure of base-denatured pepsinogen fails to give rise to a new amino terminus. Possibly, as suggested by Ong and Perlmann (9), exposure of the precursor to an acid pH brings about a conformational change which ultimately results in self-proteolysis. It is not clear, however, whether the unproteolyzed zymogen is also capable of hydrolysing other substrates (i.e. hemoglobin) or whether self-proteolysis is a prerequisite for appearance of proteolytic activity.

Although other studies have shown that the conformation of pepsin differs markedly from that of pepsinogen (6, 8), our studies indicate that Sepharose-bound pepsinogen retains most of its antigenic determinants after exposure to acid. Possibly, the binding of the pepsin portion of pepsinogen to Sepharose stabilizes the original conformation. The presence of activation peptides on the Sepharose may further stabilize the native conformation of pepsinogen.

The zymogen-enzyme transformation of pepsinogen resembles that of streptococcal proteinase (23) and that of prochymosin (prorennin) (24). In all these cases the autocatalytic activations do not require the presence of active proteolyzed species; the unproteolyzed zymogens have the ability to cleave themselves. As suggested before (25, 26), it is possible that other enzymes, for example, papain (3), are derived from precursors in a similar manner.

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