A Spectrophotometric Investigation of the Pepsinogen-Pepsin Conversion

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SUMMARY

A spectrophotometric assay of the conversion of pepsinogen into pepsin is described. This assay allows accurate investigations at early stages in the reaction which demonstrate that the conversion is more complex than a simple autocatalytic process and that a unimolecular self activation may also be involved. Evidence is presented for a rapid reversible conformational change in the pepsinogen molecule to what may be an intermediate in the conversion.

The kinetics of the pepsinogen-pepsin conversion were first investigated by Herriott (1), who showed that the process is autocatalytic, i.e., the product of the reaction catalyzes further conversion. More recently, Bustin and Conway-Jacobs (2) presented evidence that the initial step in the process is an intramolecular cleavage of the zymogen.

Most investigations of this conversion have been performed by assaying the appearance of pepsin activity by some variant of the Anson assay procedure (3), a technique which precludes the investigation of the early time course of the reaction. By measurement of optical rotatory dispersion, Perlmann (4, 5) has demonstrated differences in conformation between pepsinogen and pepsin, while Teale and Badley (6) have shown that 1 or 2 tryptophan residues which are buried in pepsinogen are unmasked and made accessible to iodide ion in pepsin. These considerations suggested that it might be possible to observe the conversion spectrophotometrically by measuring changes in tryptophan absorption. This report shows that it is possible to follow the conversion in this way and also demonstrates a rapid reversible conformational change to what may be an intermediate in the reaction.

EXPERIMENTAL PROCEDURE

Materials—Pepsinogen (lots PG 1RA, PG 1GB, and PGC 11A) and pepsin (lot PM 1BA) were obtained from Worthington. Concentrations of these proteins were measured using a molar extinction coefficient of $5.1 \times 10^4$ at 280 nm. Sperm whale myoglobin was from Sigma. All other reagents were analytical grade or equivalent.

Methods—Pepsinogen was dissolved in 2 mM Tris chloride, pH 8.0, and dialyzed against this solvent overnight. This solution was brought to the required initial concentration, pH, and ionic strength by addition of 2 mM Tris chloride or of 1 M sodium chloride in 2 mM Tris. The conversion was initiated by the addition of 25 µl of 0.8 M sodium formate buffer or of small aliquots of concentrated perchloric acid to 2 ml of pepsinogen solution in a spectrophotometer cuvette using an “add-a-mixer” obtained from Precision Cells, Inc. The resulting absorbance changes were followed as a function of time in a Cary model 15 spectrophotometer using the 0 to 0.1 absorbance scale. Difference spectra were measured in the same instrument. The final pH of the solution was measured in the cuvette using a Radiometer pH meter 26 with a GK 2321 C combined electrode. All experiments were carried out at 25°C.

Pepsin activity was measured by the method of Anson (3), except that denatured myoglobin was used as the substrate.

Analysis of Kinetics—To facilitate the presentation of results, the model used to analyze the kinetics of the absorbance changes resulting from the conversion will be presented here.

The results of Herriott (1) and Bustin and Conway-Jacobs (2) may be combined into a general model in which the conversion of pepsinogen (A) into pepsin (B) may occur unimolecularly, as in Equation 1,

$$ A \xrightarrow{k_1} B $$

or by an autocatalytic bimolecular process,

$$ A + B \xrightarrow{k_2} 2B $$

When both of these processes can occur then the rate of disappearance of pepsinogen,

$$ \frac{d(A)}{dt} = - k_1 \cdot (A) - k_2 \cdot (A) \cdot (B) $$

If the initial concentrations of pepsinogen and pepsin are $A_0$ and $B_0$, respectively, then

$$ -(k_1 + k_2(1) \cdot (B_0)) t = \ln \left( \frac{(A)}{(A_0)} \cdot \frac{k_1 + k_2(1) \cdot (B_0)}{k_1 + k_2(1) \cdot (B)} \right) $$

If the unimolecular reaction does not occur and the initial con-
FIG. 1. Difference spectra of pepsinogen solutions. A, sample, 20 \mu M pepsin in 2 mM Tris, pH 7.5; reference, 20 \mu M pepsinogen in 2 mM Tris, pH 7.5. B, sample, 20 \mu M pepsinogen, in 2 mM Tris, 10 mM sodium formate buffer, pH 4; 90 min after acidification; reference, 20 \mu M pepsinogen in 2 mM Tris, pH 7.5.

FIG. 2. Kinetics of the absorbance change at 287.5 nm following the acidification of pepsinogen solutions from 2 mM Tris, pH 7.5, by the addition of sodium formate buffer to a final concentration of 10 mM, pH 4. (i), 20 \mu M pepsinogen; (ii), 20 \mu M pepsinogen, 2.5 \mu M pepsin; (iii), 20 \mu M pepsinogen, 10 \mu M pepsin.

centration of pepsin is small, then

\[-k_2 \cdot (A_0) \cdot t - \ln \left( \frac{(A)}{(B)} \cdot \frac{(B_0)}{(A_0)} \right)\]  \hspace{1cm} (5)

which is Horratt's original equation. On the other hand, if there is no pepsin initially present, or if it has been irreversibly denatured, then

\[-(k_1 + k_2 \cdot (A_0)) \cdot t = \ln \left( \frac{(A)}{(B)} \cdot \frac{k_1}{k_2 \cdot (A_0)} \right)\]  \hspace{1cm} (6)

After the reaction has proceeded for a considerable extent, \(k_1 (B) \gg k_2,\) and

\[-(k_1 + k_2 \cdot (A_0)) \cdot t = \ln \left( \frac{(A)}{(B)} \cdot \frac{k_1}{k_2 \cdot (A_0)} \right)\]  \hspace{1cm} (7)

Equation 7 is analogous to Equation 5 and predicts that a semilogarithmic plot of the ratio of pepsinogen to pepsin, against time, will eventually become linear. The slope of this linear portion is \(k_1 + k_2 \cdot (A_0)\) and the intercept at \(t = 0\) is \(k_2 \cdot (A_0)/k_1.\) Such plots may be used to evaluate the two rate constants and will be used for this purpose throughout the paper.

RESULTS

Demonstration of Difference Spectrum between Pepsin and Pepsinogen—Fig. 1 shows the difference spectrum recorded with a solution of pepsin in the sample cuvette and a solution of pepsinogen in the reference cuvette. Both solutions were 20 \mu M in protein, 2 mM Tris chloride, pH 7.5. To show that this difference spectrum was indeed due to the conversion of pepsinogen to pepsin, the reference solution was adjusted to pH 4 by the addition of formate buffer and the difference in absorbance at 287.5 nm was followed as a function of time. After a lag period of about 700 s, the difference began to decrease. Fig. 2 shows the variation of the absorbance difference as a function of time. The variation is sigmoidal in form, characteristic of an autocatalytic process, and is greatly accelerated by the addition of increasing concentrations of pepsin to the reference solution. When no further change in absorbance could be detected, the acidified pepsinogen solution was transferred to the sample compartment and a suitably diluted neutral solution placed as reference. Fig. 1 shows the difference spectrum recorded between these solutions. The difference was not lost when the acid solution was again neutralized with Tris.

In Fig. 3 the kinetics of the absorbance change are analyzed as a semilogarithmic plot of the ratio of pepsinogen \((A)\) to pepsin \((B)\). It will be seen that the plot is initially curved, but becomes
linear after 1500 s. The dashed line was fitted to these points and used to calculate the rate constants, $k_1$ and $k_2$, from Equation 7. The solid line was calculated from Equation 6 using the derived values.

A similar experiment performed on a solution of pepsin, under the same conditions of concentration, ionic strength, and pH produced no change in absorbance over a comparable period of time.

To show that the change in absorbance was actually associated with the conversion reaction, the following experiment was performed. First, 10 ml of a 20 μM solution of pepsinogen in 0.1 M sodium chloride, 2 mM Tris chloride, pH 7.5, were brought to pH 4.5 by the addition of formate buffer. Then, a sample was transferred to a spectrophotometer cuvette and the absorbance at 287.5 nm was followed as a function of time. At intervals of 1000 s, 0.5-ml samples were removed from the remainder of the solution, adjusted to pH 8.5 with dilute sodium hydroxide, and diluted to a total volume of 10 ml. These dilutions were then assayed against acid-denatured myoglobin. The time dependence of the absorbance change and of the appearance of pepsin are shown in Fig. 4.

Effect of Ionic Strength—Herriott has demonstrated that the rate of the conversion reaction is a marked function of ionic strength and pH (1). Fig. 5 shows a series of semilogarithmic plots, following the variation of the absorbance change as a function of time, at a fixed pH of 4.02 with increasing sodium chloride concentration. Only the linear portions of the plots are shown in order to avoid confusion.

Effect of pH—The composition of the formate buffer added to the neutral pepsinogen solution was varied to produce a final pH of 4.5 or less. Fig. 6 shows a series of semilogarithmic plots, derived from these experiments, following the variation of the absorbance change as a function of time at fixed ionic strength but with decreasing pH.

Possible Intermediate in Reaction—As the pH of the reaction mixture was progressively lowered below pH 4, it became clear that an increasing fraction of the absorbance change occurred within the mixing period of the pH change (2 to 3 s) and that this was then followed by another slow absorbance change. A study of the pH dependence of this fast change and of the kinetics of the slow change was made difficult by the fact that in the majority of experiments the fast loss of absorbance was followed by a slow increase, the latter resulting from transient precipitat-
The precipitation (which has not been previously reported) is probably not involved in the conversion reaction, since in the few experiments in which it did not occur, the absorbance changes and the production of pepsin activity proceeded as expected. It seems likely that the precipitation was due to the fact that the experiments were performed close to the isoelectric pI of pepsinogen (pI 3.7). Once the protein had been converted to pepsin, which has an isoelectric point below pI 1, it redissolved.

To investigate the pH dependence of the fast change of absorbance, the solution was made 0.25 M in sodium chloride and 2 μM in pepsinogen. Both of these maneuvers served to reduce the rate and extent of the precipitation. The low concentration of protein made it necessary to follow the absorbance change at 235 nm (in other experiments, changes at this wavelength always paralleled those at 287.5 nm). Since formate absorbs in this wave length region, pH changes were made by the addition of predetermined aliquots of perchloric acid. Kinetic curves from the spectrophotometer were extrapolated back to zero time to give the magnitude of the fast change. This change was found to be a sigmoidal function of pH with a midpoint at approximately pH 3.5.

In experiments in which precipitation did not occur, difference spectra were recorded to indicate the nature of the regions of the molecule involved in the two changes. Fig. 7 shows a series of difference spectra recorded in such an experiment. For Spectra A and B, the reference cuvette contained a 20 μM solution of pepsinogen in 2 mM Tris chloride, pH 7.5. The sample cuvette contained a similar solution which had been adjusted to pH 2.85. The difference spectra were recorded after 1 min (Spectrum A) when essentially no conversion to pepsin had occurred, and after 60 min (Spectrum B) when the conversion was complete. For Spectrum C, the sample solution had been at pH 2.85 for 70 min and the reference solution, for less than 2 min.

To test the reversibility of the absorbance changes, the pepsinogen solution was taken to pH 2.85 and then neutralized again to pH 7.3 by the addition of Tris. Fig. 8 shows difference spectra recorded when the sample solution was kept at pH 2.85 for less than 2 min (Spectrum A) and for 75 min (Spectrum B).

**DISCUSSION**

The difference spectra presented here indicate that the conversion of pepsinogen to pepsin is accompanied by a change in the environment of some of the aromatic residues in the molecule. The peaks at 295 nm and at 281 nm are characteristic of the perturbation of tryptophans (in agreement with the results of Teale and Badley (6)), while the large peak at 287.5 nm (where a trough is normally found in tryptophan difference spectra) clearly indicates a sizeable change in the environment of a number of tyrosine residues, a fact which would not have been observed in fluorescence measurements. Using the semiempirical approach suggested by Donovan (8), the conformational change giving rise to the observed difference spectrum could be analyzed to indicate the complete exposure of as few as 1 tryptophan and 2 tyrosine residues. Alternatively, smaller changes in the environment of a larger number of residues may be involved.

When a solution of pepsinogen is brought to conditions where it is converted into pepsin, a similar difference spectrum is developed. Where comparable, the magnitude of this difference spectrum parallels the appearance of pepsin and shows similar dependence on the concentration of added pepsin, on ionic
strength, and on pH. This suggests that the change in absorbance is associated with a step in the conversion process and may be used as a convenient parameter to follow the kinetics of the process. The advantages of such a continuously observable parameter over any measurements based on an assay of pepsin activity are self-evident.

It is disturbing that the difference spectra generated in the kinetic experiments reported here are not identical with that measured between commercial pepsin and pepsinogen. Indeed, the difference spectra seem to vary slightly among themselves, depending on the conditions under which the conversion took place. Rajagopalan, Moore, and Stein (9) have demonstrated that the homogeneity of pepsin preparations depends markedly on the pH at which the conversion occurred. The variation of the difference spectra may reflect this factor.

The results shown in Fig. 3 demonstrate that at an early stage, during which absorbance changes can be measured more accurately than enzyme activity, the kinetic results presented here are in agreement with the postulate of Bustin and Conway-Jacobs (2), i.e., that the initial formation of pepsin occurs by a unimolecular cleavage of pepsinogen. However, it is easier to calculate the rate constants for the unimolecular process, and hence, only the sections of the results are presented here.

It must be pointed out that the good agreement of the experimental data to Equation 6 cannot be taken as proof that the initial step in the conversion reaction is a unimolecular self-cleavage of the zymogen. Very similar equations can be derived if it is assumed that the initial step is a bimolecular reaction between 2 molecules of pepsinogen, which produces 1 or 2 molecules of pepsin. To distinguish between these three situations would require careful analysis of kinetic curves obtained over a wide range of protein concentration. The numerical values of the rate constants obtained in all three of these cases are very similar, although the units and consequently any interpretation of k2 would be different if the self activation of pepsinogen were a bimolecular process. The results of Bustin and Conway-Jacobs suggest that this is not the case.

Curves ii and iii in Fig. 2 could be fitted to Equation 4 reasonably well. However, the fit was improved by using slightly different values of the rate constants from those determined in Fig. 3. This presumably resulted from the small differences in pH between the different solutions and differences in specific activity of the added pepsin and that formed in the reaction.

Fig. 5 shows that both rate constants are functions of ionic strength. More interestingly, the constancy of the intercept of the linear plots at the ordinate (= k2/(As/k2)) shows that at pH 4.02, k2 and K1 have parallel variation with ionic strength. This suggests that the same step is rate-limiting in both modes of activation. Plots of log k2 and log k1 against 1/2 fall on parallel curves of decreasing slope. The limiting slope of the rates at low ionic strength is 4, indicating that the rate-limiting step in each case involves the separation of two species of high and opposite charge. This is in agreement with Herriot's suggestion that, in this pH range, the rate-limiting step in the production of pepsin activity is the separation of the positively charged inhibitor peptide from the negatively charged protein (1).

The rate constants are marked functions of pH and both increase as the solution becomes more acid. However, their behavior is complex. Both depend on more than the first power of the hydrogen ion concentration suggesting that the conversion requires the protonation of several basic groups in the protein. This complexity, together with the experimental difficulties mentioned above, has prevented a complete analysis of the pH dependence of the rate constants which would clearly indicate that the rapid conformational change, detected below pH 4, does indeed represent an intermediate in the conversion reaction. However, an examination of the difference spectra shown in Figs. 7 and 8 suggests that this conclusion has some merit.

Acidification of a solution of pepsinogen to pH 2.85 produces a rapid conformational change, which is completely reversible on renaturation and is not accompanied by the formation of pepsin. If the solution is maintained at this pH, further changes occur, as shown by slow changes in the shape and magnitude of the difference spectrum. A difference spectrum between a solution which has been held at acid pH for some time and one which has been freshly acidified suggests that these changes affect only tyrosine residues. If the solution is now renatured the absorbance change is only partially reversed to give the difference spectrum between pepsin and pepsinogen which is observed above pH 4.

It is tempting to interpret the fast change as a pH-dependent conformational change which brings the molecule into a state where irreversible bond cleavage can occur. At higher pH this step would be rate-limiting and the two absorbance changes would occur together. An alternative explanation, of course, is that the fast change is a side reaction which is not involved directly in the activation process. Blumenfeld and Perlmann (10) have shown that the near ultraviolet spectrum of pepsin is a function of pH, below pH 4, and it seems likely that the partial reversibility of the absorbance change reported here is reflecting this variation. The present experiments do not distinguish between these two situations.

Acknowledgment—I would like to thank Dr. Harold Edelhoch for a helpful discussion.

REFERENCES

A Spectrophotometric Investigation of the Pepsinogen-Pepsin Conversion
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