The Autoactivation of Trypsinogen*

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SUMMARY

The ability of trypsinogen to catalyze its own activation was studied at pH 8.1 in the presence of Ca\(^{++}\) ions. The lag phase portion of the classical S-shaped curve of activation was shortened by increasing the trypsinogen concentration but was extended by pretreatment of the zymogen with diisopropyl fluorophosphate or soybean trypsin inhibitor. Despite these pretreatments, however, full activation was achieved.

In confirmation of the proposed catalytic activity of trypsinogen, acetyltrypsinogen, which cannot be activated by trypsin or acetyltrypsin, was able to activate chymotrypsinogen at a rate about 10\(^{-6}\) that of trypsin and to hydrolyze \(\beta\)-toluenesulfonyl arginine methyl ester at a very slow rate. Pretreatment of the zymogen with diisopropyl fluorophosphate and soybean trypsin inhibitor did not affect the rate at which it carried out these two reactions, thus excluding the presence of trypsin or acetyltrypsin.

The conclusion is drawn that the activities observed were not due to contamination of the zymogen preparations with active enzyme but were due to an activity inherent in the zymogens. The S-shaped curve of trypsinogen activation is now interpreted as two separate phases: a slow initial formation of trypsin by self-activation of the zymogen, followed by a rapid acceleration of the reaction due to catalysis by the product, trypsin.

The transformation of bovine trypsinogen into trypsin is effected by release of a single peptide, Val-(Asp)4-Lys, from the amino terminus of the zymogen (2) accompanied by a conformational change (3, 4). This reaction is accelerated by trypsin, particularly in the presence of Ca\(^{++}\) ions (5, 6). It has always been assumed that the activation of trypsinogen is initiated by a small amount of contamination by trypsin. This does not adequately explain how the first molecule of trypsin was formed.

It is known that trypsinogen can also be activated by entero-kinase (7) but since trypsinogen is purified from pancreatic extracts and entero-kinase originates in the duodenal mucosa, activation by this route does not enter into in vitro studies.

Trypsin is essential for the activation of other proteolytic zymogens, for example, chymotrypsinogen and procarnoylpeptidase A. The formation of this important enzyme from its zymogen precursor was studied in an attempt to determine whether the zymogen itself might have inherent proteolytic activity, enabling it to activate itself.

METHODS

Materials—Bovine trypsinogen (crystallized once, Lot TG OGL), bovine chymotrypsinogen A (crystallized five times, Lot CGC OEA), bovine trypsin (crystallized three times, Lot TRL 7EC), and soybean trypsin inhibitor (Lots SI 618 and 7DB) were purchased from Worthington. The zymogens were dialyzed against 1 M HCl and lyophilized before use. The trypsin was further purified on SE-Sephadex by the method of Papioannou and Liener (8). DFP* was a gift from Merck, Sharp and Dohme. The trypsin substrate, \(\beta\)-toluenesulfonyl-L-arginine methyl ester, was synthesized by standard methods (9) and benzoyl-L-tyrosine ethyl ester (Lot D4304), the chymotrypsin substrate, was a product of Schwarz/Mann, Orangeburg, New York. All other chemicals were of reagent grade, and deionized water was used throughout.

Activity Measurements—Trypsin and chymotrypsin were determined spectrophotometrically with TAMe and BTEe as the respective substrates (10). One unit represents the hydrolysis of 1 \(\mu\) mole of substrate per min. A divided cell was used for the blank with protein in one half and substrate in the other.

Activation Experiments—Trypsinogen for activation was prepared by suspending a weighed amount of the zymogen in 1 M HCl and centrifuging off the insoluble material. Protein concentrations were determined by using \(A_{280}\) of 15.4 for trypsinogen, 20.4 for chymotrypsinogen, and 9.1 for soybean trypsin inhibitor. Activation runs were conducted by introducing a measured amount of trypsinogen or acetyltrypsinogen solution into 0.1 M Tris-HCl buffer, pH 8.1, containing 0.05 M CaCl\(_2\) and 0.01 M indole (a chymotrypsin inhibitor in the experiments that did not involve chymotrypsinogen). Other materials were then added as necessary, and the incubations were performed at 30 or 32°C. Samples were removed for assay versus TAMe or BTEe at various intervals.

Preparation of Acetyltrypsinogen—Trypsinogen (195 mg) was dissolved in 40 ml of 0.01 M Tris-HCl, pH 7.6, chilled to 4°C, and 40 ml of redistilled acetic anhydride were added. The pH was maintained at 7.6 by the addition of 0.1 M KOH, controlled by an autotitrator (Radiometer type TTT 11b). After approximately 30 min at 4°C, the addition was complete, and the clear* This research was supported by the National Science Foundation (GB-12630) and by the United States Public Health Service (Grant AM-09826 from the National Institute of Arthritis and Metabolic Diseases). A preliminary report has been published (1).
solution was dialyzed against 1.8 mM acetic acid. The acetylated zymogen precipitated in the bag. The contents of the bag were emptied out, and 5 ml of 0.1 M Tris-HCl, pH 7.8, were added to the suspension of the well-stirred protein; the pH was adjusted to 7.6 with KOH, and the protein redissolved. The acetylation and dialysis steps were repeated. Finally, the acetic acid suspension was lyophilized. The yield of acetyltrypsinogen was 189 mg, and this material could not be activated even on addition of trypsin.

**Treatment of Trypsinogen and Acetyltrypsinogen with DFP**—In a typical experiment, 20 mg of zymogen (either trypsinogen or acetyltrypsinogen) in 1.0 ml of 0.1 M Tris-HCl, pH 8.1, were divided into two lots. One sample served as a control, while to the other 20 μl of a freshly prepared solution of DFP (0.05 M in 1.2 ml of isopropanol) were added. Both samples were left at either 0° in the case of trypsinogen or room temperature for acetyltrypsinogen for 1 hr and then acidified to pH 4. The control sample was left in the refrigerator overnight, while the treated sample was dialyzed against 0.1 mM HCl with one change. Both samples were then readjusted to pH 8 with NaOH, and the protein concentration was determined spectrophotometrically.

**RESULTS**

**Activation of Trypsinogen**—Incubation of trypsinogen under the usual conditions (defined under “Methods”) led to complete activation of the zymogen without the addition of exogenous trypsin. A classical S-shaped profile (11) was obtained with an initial lag period during which no tryptic activity with respect to TAME could be detected. The length of this lag phase (defined as the time required for the first appearance of detectable tryptic activity) increased from approximately 30 to 3600 min on decreasing the trypsinogen concentration from 7.2 to 0.1 mg per ml (Table I). Activation in the presence of Ca++ proceeded equally well in an unbuffered solution, the pH being maintained constant at 8.1 by means of the autotitrator; this ruled out any effect of buffer ions on the activation.

The development of activity can be explained by one of two possibilities: (a) the trypsinogen preparation contains some endogenous trypsin at a level below the limits of detection of the TAME assay, or (b) trypsinogen itself has an inherent activity that slowly generates trypsin by an intra- or intermolecular reaction during the lag phase and then, once trypsin is formed, the activation accelerates rapidly. The following experiments were conducted in an effort to resolve this problem.

**Activation of DFP-treated Trypsinogen**—DFP-treated trypsinogen was prepared as described under “Methods” under conditions that inactivated 60 μg of trypsin to a level where no activity whatsoever could be detected with TAME. Aliquots (trypsinogen concentration finally 3.0 mg per ml) were diluted in activation buffer, and the activation was followed at 32°. The lag times were approximately 10 min for the control sample as against 10 min for the DFP-treated sample, but full activation was achieved in both cases. Thus, even if there is some trypsin in the trypsinogen preparation, treatment with such a vast excess of DFP should inactivate most, if not all, of the enzyme. The longer lag phase for the treated sample may indicate the presence of a minute trace of trypsin, but the major point to be established at this juncture is that activation still occurs even after this DFP treatment.

**Activation of Soybean Inhibitor-treated Trypsinogen**—Before an attempt could be made to remove any active trypsin from the activation mixtures with soybean inhibitor, it had to be shown that trypsinogen would not interact with the inhibitor. This was done in two ways.

1. After incubation of 11 mg of trypsin and 5 mg of inhibitor in 1 ml of 0.01 M Tris-HCl, pH 7.7, containing 0.05 M CaCl₂ for 15 min at room temperature, the mixture was applied to a column of Sephadex G-75 (0.9 × 160 cm), equilibrated with the same buffer. The column was operated under gravity at 10°, and 1.3-ml fractions were collected. Two peaks were obtained, corresponding to the trypsin-inhibitor complex and free trypsin (Fig. 1, upper). In contrast, when the experiment was repeated, substituting trypsinogen for trypsin, only one peak was found (Fig. 1, lower), as would be expected from the similar sizes of trypsinogen and the inhibitor if no complex formation had occurred. An identical profile to the lower curve was obtained when trypsinogen alone was chromatographed.

**Table I**

*Activation of trypsinogen in the presence and absence of soybean trypsin inhibitor*

At each concentration of zymogen, incubation with or without inhibitor (molar ratio of zymogen to inhibitor of 200:1) was conducted at 32° in 0.1 M Tris, pH 8.1, containing 0.06 M Ca++. 

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<th>Trypsinogen</th>
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* Defined as the time for the first appearance of detectable tryptic activity.

Fig. 1. Gel filtration on Sephadex G-75 of a mixture of 11 mg of trypsin and 5 mg of soybean inhibitor (upper) and a mixture of 14 mg of trypsinogen and 6 mg of inhibitor (lower). The column was 0.9 × 160 cm and was operated at 10°; the buffer was 0.01 M Tris-HCl, pH 7.7, containing 0.05 M CaCl₂. Fractions were 1.3 ml, and the flow rate was 18 ml per hour. —, A₄₅₀; --, tryptic activity versus TAME.
2. The "competitive assay" method of Feinstein and Feeney (12) was used. In a typical experiment, 8.9 μg of soybean inhibitor in 2.7 ml of 0.05 M Tris-HCl, pH 8.1, containing 0.02 M Ca++, were incubated for a period of time (usually 10 min) with varying amounts of zymogen at 32° in a 3-ml cuvette. At this time, 4.3 μg of trypsin (in 20 μl of 1 mM HCl) were added, and an additional 1 min was allowed for it to combine with the inhibitor remaining. TAME (0.3 ml) was then added to determine the tryptic activity remaining. Control incubations showed that there was no activation of the trypsinogen by the trypsin during the 1 min in which they were in contact. It is evident from Fig. 2 that trypsinogen does not interfere with the reaction between trypsin and soybean inhibitor as long as the ratio of zymogen to inhibitor is kept below approximately 300/1. This was true even on incubation for 30 min in this range of concentration.

Thus, activation incubations could be set up at different concentrations of trypsinogen with the ratio of zymogen to inhibitor always 300/1. Table I shows that inclusion of the inhibitor extended the lag phase in each case, but full activation was always achieved. Thus, either the inhibitor is removing some endogenous trypsin, hence prolonging the lag time; or, if no trypsin is present, the first few molecules of free trypsin produced by self-activation of the zymogen are taken up by the inhibitor into the trypsin-inhibitor complex. These trypsin molecules are not then available to re-enter the activation process and accelerate it.

These experiments suggested that trypsinogen catalyzed its own activation, but, to demonstrate, unequivocally, catalysis by the zymogen (if it existed) a system was needed in which the advantage in these experiments was that, if any activity due to acetyltrypsin was present, the level had to remain constant since acetyltrypsinogen cannot be activated. Treatment of the acetyltrypsinogen with DFP should remove some if not all of the acetyltrypsin, and therefore a significant difference should be seen between treated and untreated samples.

Aliquots of acetyltrypsinogen were treated with DFP at room temperature as described under "Methods." Triplicate samples (5.5 mg) of both treated and control solutions were incubated with 19 μg of inhibitor at pH 8.1 in 0.1 M Tris-HCl containing 0.05 M Ca++ for 5 min before the addition of 12 mg of chymotrypsinogen (in 0.25 ml of 1 mM HCl) to adjust the final volume to 3.0 ml. Incubation was carried out at 30°, and samples were removed for assay of chymotryptic activity against BTEE. Fig. 3 shows a linear increase in specific activity for both treated and control samples. No trypsic activity versus TAME was found after 100 hours of incubation, and no increase in chymotryptic activity occurred in the control without acetyltrypsinogen. The lines drawn in Fig. 3 were calculated by the method of least squares, and the slope for the DFP-treated and control samples were 0.008 ± 0.001 and 0.007 ± 0.001, respectively (not a significant difference). The intercepts were not zero because the chymotrypsinogen preparation had a small amount of free chymotrypsin in it. Complete activation of the chymotrypsinogen present would have produced a final specific activity of 27 units per mg. In this experiment the chymotrypsinogen present was thus activated at the rate of about 1% per day; in another experiment (not shown) the rate was slightly faster.

Thus, treatment of acetyltrypsinogen with DFP did not alter

![Fig. 2. Interaction of trypsinogen with soybean inhibitor.](http://www.jbc.org/)

![Fig. 3. Activation of chymotrypsinogen A by acetyltrypsinogen.](http://www.jbc.org/)
soybean inhibitor (16: and 8 pg, respectively) for 5 min. TAMe Tris-HCl, pH 8.1, containing 0.02 trypsinogen (0, 0, 1 mg per ml; El, 0.5 mg per ml) in 0.05 other side of a divided cuvette. The extinctions were measured at 247 nm against a blank containing protein in one side and TAMe in the other side of a divided cuvette. DFP, DFP-treated; O, □, untreated.

the rate at which this modified zymogen activated chymotrypsinogen. Hence, the “trypsin” activity found was not due to contamination by free enzyme but was due to an inherent activity of the acetyltrypsinogen. A rough calculation from the rate of activation of chymotrypsinogen by acetyltrypsinogen shows that acetyltrypsinogen has approximately $10^{-5}$ the activity of trypsin.

Hydrolysis of TAMe by Acetyltrypsinogen—To check for catalytic activity on another substrate, it was thought that the acetyltrypsinogen might digest TAMe if given a long enough time to do so. Consequently, a sample of acetyltrypsinogen was treated with DFP as already described, and triplicate incubations were set up at two different concentrations of zymogen (as given in the legend to Fig. 4). The hydrolysis of TAMe proved to be very difficult to measure since the readings at 247 nm were very heavy precipitation. The product absorbs at 405 nm, where the protein has no absorbance. Unfortunately, addition of this compound (1 mg/ml in water) was then added to bring the final volume to 3.0 ml. The extinctions were measured at 247 nm against a blank containing protein in one side and TAMe in the other side of a divided cuvette. DFP, DFP-treated; □, □, DFP untreated.

There was not a great deal of difference between the DFP-treated and untreated samples. This experiment was repeated a number of times, and the results, although not lending themselves to quantitative interpretation, all pointed to hydrolysis of TAMe by acetyltrypsinogen. These have to be intermolecular reactions.

In an effort to surmount the problem of the high blank, benzoyl arginine p-nitroanilide was tested because its hydrolysis product absorbs at 405 nm, where the protein has no absorbance. Unfortunately, addition of this compound (1 mg/ml in water) to a clear solution of acetyltrypsinogen (0.5 mg/ml in 0.1 M Tris buffer) brought about a very heavy precipitation. The reason for this is not clear.

**DISCUSSION**

In the experiments with trypsinogen, doubt remained whether the treatments used had removed every last molecule of active trypsin. It was calculated that the degree of contamination of the zymogen preparation by trypsin, if there was any, was less than 0.01% (from the rate of hydrolysis of TAMe by 1 mg of zymogen). However, since it was subsequently found that acetyltrypsinogen could hydrolyze TAMe, this figure is probably far too high. DFP treatment (using approximately a 30,000-fold molar excess over the calculated 0.01%) although it would have reduced any active trypsin present to a very minute level, might possibly still have left a significant number of active molecules. Similarly, treatment with soybean inhibitor, which has a $K_i$ for trypsin of around $10^{-16}$ M (16), should have drastically reduced the number of active molecules, but again an uncertainty existed whether the very last molecule had been eliminated. If even 1 molecule of active trypsin was present, then the inherent activity of trypsinogen would be in doubt.

The trypsinogen studies were complicated because the activation was composed of two phases: (a) supposedly, the zymogen acting on itself to produce trypsin and (b) this newly formed trypsin then accelerating the activation enormously.

By making use of acetyltrypsinogen, the acceleration phase was removed, because of the inability of acetyltrypsinogen to be activated. Any activity detected, either from contamination by acetyltrypsin or from activity inherent in the zymogen, thus had to produce linear effects. Since the activation of chymotrypsinogen by acetyltrypsinogen proceeded equally well whether or not the zymogen was pretreated with DFP and inhibitor, the conclusion has to be drawn that the acetyltrypsinogen (and so presumably trypsinogen) did not contain any active enzyme. Otherwise, the DFP and inhibitor treatment would surely have lowered the rate of reaction to some extent.

It can therefore be concluded that trypsinogen does have an activity inherent in itself by which it can self-activate, albeit at a very much slower rate than the activation catalyzed by trypsin. This self-activation is probably an intramolecular reaction, since the lag time increased on decreasing the concentration of trypsinogen (Table 1). This idea is lent further support by the activation of chymotrypsinogen and the hydrolysis of TAMe by acetyltrypsinogen. These have to be intermolecular reactions.

It would thus appear that trypsinogen (or acetyltrypsinogen) can carry out, at a very much lower rate, three normally trypsin-catalyzed reactions. The apparent inactivity of the zymogen to DFP may simply mean that the rate of reaction of trypsinogen with DFP is too slow to be measurable.

It is not difficult to envisage a scheme (analogous to that proposed for ribonuclease (17)) for a bimolecular interaction in which two trypsinogen molecules could come together momentarily, perhaps with conformational alterations, with one contributing the imidazole group of histidine-46 and the other the hydroxyl group of serine-183. This dimerization would form a trypsin-like active site in an occasional molecule, which could then split off the activation peptide from one or the other of these molecules or possibly from a third trypsinogen molecule to form active trypsin. This hypothetical mechanism differs from that proposed for trypsin since the positive charge on the a-amino group of the terminal isoleucine, possibly involved in maintaining the active conformation (18, 19), is not present in the zymogens. However, doubts have begun to arise that this group is essential for the activity of chymotrypsin (20, 21) (and so probably trypsin). Whether or not such a scheme might be feasible will not be known until the details of the three-dimensional structure of trypsinogen become available.

An equally likely alternative hypothesis would be that a trypsinogen molecule undergoes a structural alteration that produces an “active species” that can then act on other “substrate” molecules of trypsinogen. This is not altogether improbable
since it has already been shown (22–24) by physical methods that trypsinogen can undergo several reversible transitions that have the effect of producing a structure more like that of trypsin.

This is not the first zymogen that has been shown to have inherent proteolytic activity. Preprocarboxypeptidase A from the spiny dogfish (25) has esterase and peptidase activity; and streptococcal proteinase (26), porcine pepsinogen (27), and calf prorennin (28, 29) also have the ability to activate themselves. 15. Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, N. H., Biochemistry, 9, 1997 (1970).

Acknowledgments—We are grateful to Dr. Michael Bustin of the Weizmann Institute for informing us about his work on the autoactivation of pepsinogen before publication, and to Mr. Joseph P. Marciniszyn, Jr., for helpful discussions.

REFERENCES
