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ACTION OF HUMAN THROMBOKINASE ON HUMAN PROTHROMBIN AND *p*-TOSYL-L-ARGININE METHYL ESTER

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SUMMARY

An enzyme, thrombokinase, has been partially purified from human plasma. Its defining property is the ability to convert prothrombin to thrombin in the absence of other cofactors. This prothrombin-converting activity is independent of residual thrombin and is inhibited by soybean trypsin inhibitor but not by DFP. Various cofactors (polylysine or calcium alone, or beef serum or phospholipid in the presence of calcium) accelerate the conversion of prothrombin by thrombokinase.

INTRODUCTION

An enzymatic conversion of prothrombin to thrombin was first suggested by MORAWITZ[1]. The early history of this concept has been well covered in the review by MILSTONE[2].

CEKADA[3], MELLANBY[4], and MILSTONE[5] observed the "spontaneous" activation of prothrombin in a semipurified state. Starting with this observation MILSTONE has isolated from bovine plasma a prothrombin-converting enzyme which needs no cofactors, although these (calcium, phospholipid, Factor V) do accelerate the reaction[6–9]. Following the terminology introduced by MORAWITZ, MILSTONE designated this material thrombokinase and the proenzyme prothrombokinase. The present investigations were done to compare the characteristics of human thrombokinase with those reported for the bovine enzyme. The authors have used the term thrombokinase, since the primary assay system, *viz.*, conversion of prothrombin to thrombin, is the classic definition of thrombokinase.

MATERIALS AND METHODS

Human thrombokinase was prepared[10] starting from acid citrate dextrose

Abbreviation: TAME, *p*-tosyl-L-arginine methyl ester.

anticoagulant, NIH Solution A, bank plasma. Prothrombokinase was adsorbed onto the barium citrate precipitate formed upon the addition of $BaCl_2$ to the plasma. The precipitate was washed, and the proteins were eluted by the addition of $(NH_4)_2SO_4$. More $(NH_4)_2SO_4$ was added to precipitate the crude proenzyme. The $(NH_4)_2SO_4$ precipitate was redissolved in H_2O to about 1/1000 of the plasma volume. The pH was adjusted to 8.5 with 1 M NaOH and the solution stored at 4° for 10 days. By this time the enzyme had been activated, inasmuch as it converted prothrombin to thrombin. While probably not the optimal method of activation this method avoids the addition of extraneous materials.

This material was dialyzed against 0.15 M NaCl–0.02 M Tris·HCl (pH 7.4) and chromatographed on DEAE-cellulose (linear gradient from 0.15 M NaCl–0.02 M Tris·HCl (pH 7.4) to 0.5 M NaCl–0.066 M Tris·HCl (pH 7.4)). The active fractions were concentrated by negative pressure dialysis and rechromatographed on Sephadex G-100, obtained from Pharmacia, Piscataway, N.J.

The preparations of thrombokinase used here had protein concentrations of about 1 mg/ml, a *p*-tosyl-L-arginine methyl ester (TAME) hydrolytic activity of about 30 μmoles per 10 min per ml (about half of which was inhibited by soy bean trypsin inhibitor), and residual thrombin activity of 10–100 units/ml.

A second type of prothrombokinase preparation has been used in some of the later experiments. After the $(NH_4)_2SO_4$ precipitation of the prothrombin and prothrombokinase, the preparation was passed through Sephadex G-25 equilibrated in 0.24 M potassium phosphate buffer (pH 6.8) and placed on a column packed with hydroxyapatite* in 0.24 M potassium phosphate. Fractions were eluted by stepwise or gradient elution with increasing concentrations of the potassium phosphate. The first fraction, eluted with 0.24 M potassium phosphate, contained neither prothrombin nor detectable thrombokinase; a middle fraction eluted with approx. 0.30 M potassium phosphate contained (after activation) the majority of the thrombokinase; and the final fraction, eluted with a concentration of 0.5 M or higher potassium phosphate, contained the prothrombin. The fraction containing the prothrombokinase was precipitated with 66% satd. $(NH_4)_2SO_4$ and then activated to thrombokinase in the manner described above.

Thrombokinase activity was eluted in two positions from the DEAE-cellulose columns. Some was eluted in the breakthrough peak while the rest of the activity was eluted after one third of the gradient had delivered. In the following experiments, only the later eluting thrombokinase has been used.

Prothrombin was prepared by three different methods. Most of the experiments were done with prothrombin made by a modification[11] of the technique of GOLDSTEIN AND ZONDERMAN[12] (IR-prothrombin). A second type of prothrombin was made in the same manner except prior to chromatography on IR-50 the material was chromatographed on hydroxyapatite as described in the section on thrombokinase preparations (HAIR-prothrombin). The third prothrombin preparation was kindly supplied by the Centre National de Transfusion Sanguine and was produced by a modification of the method of SOULIER[13] (CNTS-prothrombin). The preparation as received contained

* Two commercial hydroxyapatite preparations were used. Material obtained from Bio-Rad (Richmond, Calif.) gave good separation but slow flow rates. "Hypatite-C" obtained from Clarkson Chemical Co. (Williamsport, Pa.) had a high flow rate but the protein peaks showed more tailing.

anti-thrombin which was removed by chromatography on DEAE-cellulose (linear gradient from 0.15 M NaCl–0.02 M Tris · HCl (pH 7.4) to 0.50 M NaCl–0.066 M Tris · HCl (pH 7.4)). The specific activity of all prothrombin preparations was 1200–1500 U.S. units per mg of protein. However, there was a marked difference in the stability of the different preparations in the absence of added thrombokinase, the CNTS-prothrombin and the HAIR-prothrombin being more stable than the IR-prothrombin. The addition of the CNTS-prothrombin to the plasma of a patient specifically deficient in Factor X* did not change the prolonged clotting time in the presence of tissue thromboplastin and calcium.

A commercial human fibrinogen (Merck, Sharp and Dohme) was used for all clotting assays. After dialyzing against 0.15 M NaCl–0.02 M Tris · HCl (pH 7.4) it was frozen as a 1% solution. Soybean trypsin inhibitor was obtained from Worthington Biochemical Corp., Freehold, N.J. TAME and DFP were supplied by Mann Biochemical Co., New York, N.Y.

Prothrombin was assayed by the method of WAGNER *et al.*[14]. The assay system was standardized with Lot B-3 U.S. standard thrombin, and 1 unit of prothrombin was defined as that amount yielding 1 unit of thrombin when maximally activated.

Thrombin was measured by adding 0.1 ml of the appropriately diluted test solution to 0.4 ml of 0.25% fibrinogen. Clotting times were converted to U.S. units by reference to a dilution curve of the U.S. standard thrombin.

TAME esterase activity was estimated utilizing an automatic titrator (Radiometer pH stat). The pH of 0.011 M TAME in 0.15 M NaCl was adjusted to 8.0 by the addition of NaOH. The test sample was added and the free acid titrated with 0.04 M NaOH. Results are expressed in terms of μmoles of base added per 10 min.

Factor X was assayed as described by DENSON[15]. Factor X-deficient plasma was obtained from Diagnostic Reagent, Ltd., Thames Oxon (Great Britain).

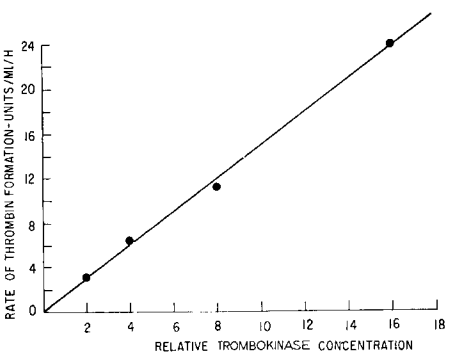

Fig. 1. The effect of thrombokinase concentration on thrombin generation. To 1.0 ml of prothrombin (4500 units/ml), in 0.02 M cacodylate–0.15 M NaCl (pH 5.95), was added 0.1 ml of dilutions of thrombokinase in 0.1 M Tris–HCl (pH 8.1). The solution was adjusted to a pH of 7.5 and incubated at 22° for 12 h. The thrombin content was measured at approx. 2-h intervals.

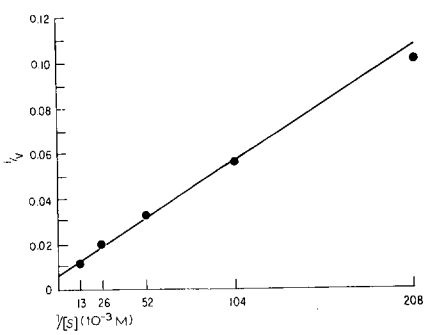

Fig. 2. Double reciprocal plot of the effect of prothrombin concentration on the rate of thrombin production by a constant amount of thrombokinase. Incubation conditions the same as for Fig. 1 except for the variation of prothrombin concentration. The units of $1/v$ are $h \cdot units^{-1} \cdot ml^{-1}$.

* This plasma was kindly supplied by Dr. O. D. RATNOFF, Cleveland, Ohio.

RESULTS

The test system for the studies on prothrombin conversion consisted of 1 vol. of prothrombin containing 4000–5000 units/ml (in 0.02 M sodium cacodylate–0.15 M NaCl (pH 5.95)) to which was added 0.1 vol. of the thrombokinase preparation (in 0.1 M Tris·HCl (pH 8.1)). The mixture (pH 7.6) was allowed to incubate for 12 h at room temperature. Aliquots were taken at various times (approx. 2-h intervals) and assayed for their thrombin content.

The velocity of thrombin generation was proportional to the thrombokinase concentration (Fig. 1). The rate of thrombin generation was constant and independent of the amount of thrombin in the incubation mixture. As shown below inhibition of the residual thrombin in the thrombokinase preparation had no effect on the conversion of prothrombin. A double reciprocal plot (Lineweaver–Burk plot) was linear over a 16-fold range of prothrombin concentration (Fig. 2).

The incubation mixtures were adjusted to various pH values in the range from 6.0 to 8.4 by addition of NaOH or HCl. They produced a change in I of less than 0.005, insufficient to change the reaction rate. The optimum pH for the reaction was 7.5 (Fig. 3).

When the reaction was allowed to proceed at a variety of temperatures, there was a striking similarity to the temperature effect reported for poly-L-lysine activation

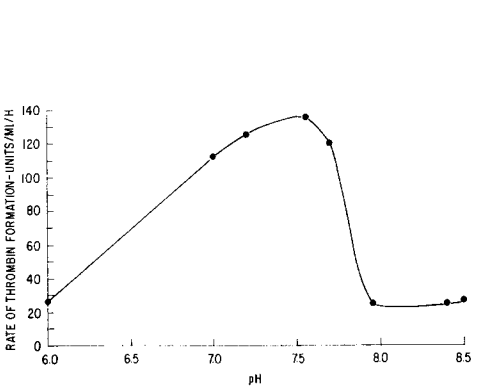

Fig. 3. The effect of pH on prothrombin conversion by thrombokinase. Incubation conditions identical with those in Fig. 1, other than adjustment of pH by addition of NaOH, or HCl.

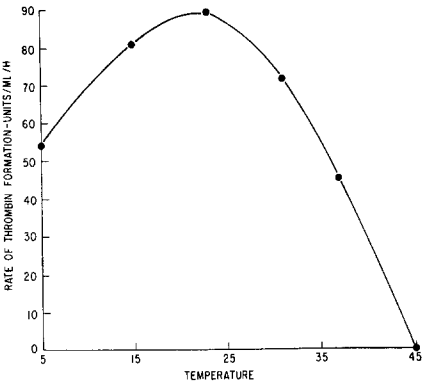

Fig. 4. The effect of temperature on the rate of thrombin generation by thrombokinase. Incubation conditions the same as in Fig. 1, except for the variations in temperature.

of prothrombin[16]. The maximum rate of thrombin generation was at 22° (Fig. 4). At the higher temperatures (28–45°) an insoluble precipitate formed in the reaction mixtures. Solutions of prothrombin alone or thrombokinase alone did not form precipitates under these conditions.

In order to evaluate the effect of ionic strength, varying amounts of solid NaCl were added to prothrombin in cacodylate which had been diluted with three parts H_2O. After incubation with thrombokinase I was adjusted to 0.16 and the samples were tested for thrombin activity. I below 0.32 slowed the reaction between thrombokinase

and prothrombin. Above 1 M NaCl there have been variable results. However, concentrations of NaCl of up to 2.5 M gave no appreciable inhibition or acceleration (Fig. 5).

Prothrombin conversion, like trypsinogen activation[17], is subject to certain specific ion effects. For many years $(NH_4)_2SO_4$ and sodium citrate at high concentrations have been found to enhance the formation of thrombin[3–5,18,19] and the paramount importance of Ca^{2+} in the whole coagulation scheme has long been recognized.

The effects of 1 M sodium acetate, NaCl and NAI on prothrombin conversion were found to be in the order reported by WARREN[20] for "denaturation" of enzymes. Acetate gave enhanced activity while iodide was inhibitory relative to chloride. On the other hand, Na^+ and K^+ effects were different from the enzyme studies by WARREN, STOWRING AND MORALES[20], with the system containing sodium acetate yielding thrombin at about twice the rate of that containing potassium acetate. (Lithium

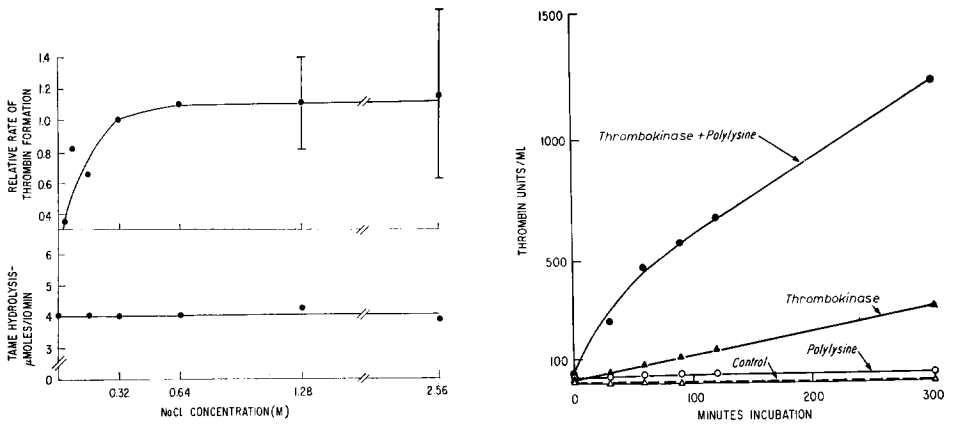

Fig. 5. The effect of I on rate of thrombin generation and TAME hydrolysis by thrombokinase. The stock prothrombin was diluted with H_2O to 1100 units/ml, and solid NaCl added to achieve indicated salt concentration. Other incubation conditions were those of Fig. 1 (upper figure). Various concentrations of NaCl were made 0.011 M in TAME and the pH was adjusted to 8.0 with NaOH. 0.1 ml of thrombokinase added to 9.0 ml of TAME solution (lower figure).

Fig. 6. Effect of polylysine on the conversion of prothrombin in the presence of thrombokinase. The incubation mixtures were: ●, 0.7 ml HAIR-prothrombin (4500 units/ml), 0.3 ml 1% polylysine, 0.1 ml of thrombokinase. ▲, 0.7 ml prothrombin, 0.3 ml H_2O, 0.1 ml thrombokinase. ○, 0.7 ml prothrombin, 0.3 ml polylysine, 0.1 ml Tris–NaCl buffer. △, 0.7 ml prothrombin, 0.3 ml H_2O, 0.1 ml Tris–NaCl buffer.

acetate had about the same activity as potassium acetate.) Because of the historical interest, 1 M citrate solutions were studied in this reaction, and the reaction rates were found to be about the same as those observed in 1 M acetate despite (a) 3 times higher cation concentration and (b) 6 times greater ionic strength. These ion effects have been studied utilizing hydroxyapatite preparations of both enzyme and substrate.

The effect of calcium on this reaction was relatively slight. The optimum concentration of $CaCl_2$ was between 0.0125 M and 0.0031 M, but raising or lowering the concentration beyond this range decreased the reaction rate by only 50%. Appropriate dilutions of the incubation mixtures were done before assaying for thrombin to eliminate the effect of the calcium on the thrombin–fibrinogen reaction.

The presence of certain polycations[16] has an effect on prothrombin conversion. Fig. 6 demonstrates that in the experimental system studied here poly-L-lysine acts as a cofactor for thrombokinase, but alone does not produce measurable amounts of thrombin. The prothrombin and thrombokinase used were prepared with hydroxyapatite.

The addition of phospholipid* to the incubation mixture to a concentration of 0.1% had no effect on the thrombin generation. In the presence of 0.0125 $CaCl_2$ there was a synergistic accelerating effect that was much further potentiated by the addition of dilute beef serum**.

Soybean trypsin inhibitor inhibited the conversion of prothrombin to thrombin. Moreover, there was no detectable change in the DEAE chromatographic pattern of the prothrombin so treated. The addition of soybean trypsin inhibitor (50 μg/ml) after thrombin generation had reached constant velocity resulted in inhibition of the reaction (Fig. 7).

Incubation of the thrombokinase with 10^{-3} M DFP followed by dialysis blocked residual thrombin activity. There was no loss of the ability to convert prothrombin.

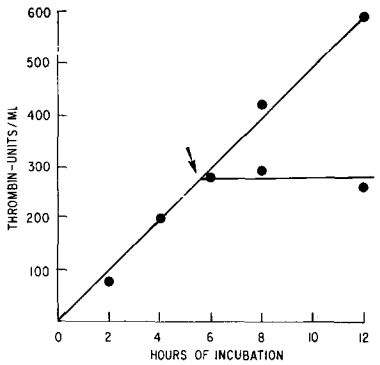

Fig. 7. Effect of soybean trypsin inhibitor added to solution of prothrombin and thrombokinase. 6 h after start of incubation of prothrombin with thrombokinase a 1-ml sample removed and added to 50 μg of soybean trypsin inhibitor (arrow).

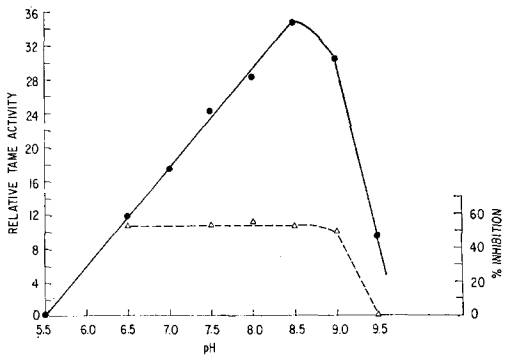

Fig. 8. Effect of pH on TAME hydrolysis by thrombokinase (●). Effect of pH on inhibition of TAME hydrolysis by soybean trypsin inhibitor, 1 mg/ml (△). 0.1 ml of thrombokinase was added to 9.0 ml of 0.011 M TAME and the pH adjusted with NaOH. The pH was kept constant by automatic titration with 0.04 M NaOH. Measurements were made at room temperature (22°).

While the defining property of thrombokinase is its ability to activate prothrombin without additional reagents, an important activity associated with the bovine thrombokinase is the ability to hydrolyze the synthetic substrate TAME[21]. The

* Inosithin: soybean phospholipid obtained from Associated Concentrates, Woodside, Long Island, N.Y.

** The addition of both Ca^{2+} and phospholipid to IR-prothrombin gave a significant rate of conversion in the absence of added thrombokinase; the CNTS-prothrombin and the HAIR-prothrombin showed much greater stability in this respect. In the presence of added thrombokinase, however, all three prothrombin preparations exhibited the same degree of acceleration by Ca^{2+} *plus* phospholipid.

human material also hydrolyzed TAME (pH optimum, 8.5) (Fig. 8). The maximum rate of ester hydrolysis was obtained at 0.03 M TAME and above.

The effects of the various salts on the hydrolysis of TAME by thrombokinase showed many similarities to those seen during prothrombin conversion. Sodium salts gave higher activities than did potassium or lithium salts; citrate and acetate gave greater activity than chloride or iodide. There was no measurable effect of NaCl from 0.0 to 2.56 M (Fig. 5).

The TAME esterase activity of the thrombokinase preparations used here was about 60% inhibitable by soybean trypsin inhibitor in the pH region 6.5–8.5 (Fig. 8). Above and below these pH's there was less inhibition of the TAME activity. The remaining TAME activity was DFP-sensitive and thus represented residual thrombin and perhaps other enzymes that hydrolyze TAME. The addition of ε-amino caproic acid to a concentration of 0.1 M did not inhibit TAME hydrolysis.

DISCUSSION

A variety of techniques have been used in the laboratory for the conversion of prothrombin to thrombin. These have made use of such substances as tissue extracts, trypsin[22], salts at high concentrations, chloroform[3], ethanol[3], polycations[16], and plasma derivatives. This diversity of reagents has given the coagulationist a diverse heritage of hypotheses for the mechanism of prothrombin conversion. MORAWITZ[1] proposed an enzymatic conversion analogous to the activation of trypsinogen. HOWELL[23] proposed an activation mechanism dependent upon the removal of heparin or an heparin-like substance from the prothrombin. MERTZ, SEEGERS AND SMITH[24] in 1939 presented evidence suggesting a stoichiometric reaction with the yield of thrombin being proportional to the amount of tissue thromboplastin added. The possibility of autocatalysis was examined by several investigators who differed in their conclusions[25,26]. The activation of prothrombin by polycations has been interpreted as a non-enzymatic process[16].

EAGLE's observation[22] that trypsin could convert prothrombin was the first direct evidence of proteolytic activation. The current evidence strongly supports the view that proteolytic digestion is sufficient for prothrombin conversion to thrombin. During the activation process, there is a marked change in the size of the prothrombin from about 65 000 to 32 000 or less[27–31]. During the conversion, there is the release of several inactive fragments[10,32]. While there is lack of agreement on the N-terminal amino acids found in thrombin, an amino acid (or amino acids) different from that of prothrombin has always been reported[33–36]. Although no C-terminal amino acid of prothrombin is detectable with carboxypeptidase[37], MAGNUSSON[38] has found a C-terminal arginine in thrombin after citrate conversion. Supportive evidence for proteolytic activation is the inhibition of prothrombin conversion by soybean trypsin inhibitor, a known inhibitor of proteolysis[39–42].

The observation that purified preparations of prothrombin "spontaneously" activate (particularly in high salt) has been interpreted in three ways: (a) activation of prothrombin is a matter of chain configurational change or dissociation, or (b) thrombin catalyzes the conversion of prothrombin, or (c) prothrombin preparations contain an enzyme or proenzyme capable of activating prothrombin.

MILSTONE[43], assuming that one of the last two interpretations was correct,

was able to achieve partial separation of the bovine prothrombin-converting proenzyme from the prothrombin. After activation of this proenzyme he was able to separate it from thrombin by a variety of methods—$(NH_4)_2SO_4$ precipitation, isoelectric precipitation, electrophoresis and chromatography[6,44–46]. These experiments lead to the conclusion that there is a plasma enzyme that converts prothrombin to thrombin and that thrombin is not necessary for prothrombin conversion. This enzyme, thrombokinase, will activate prothrombin with no additional material. The addition of calcium and phospholipid and/or adsorbed beef serum accelerates the reaction, although phospholipid or beef serum alone does not[6–9]. A different approach led MARCINIAK AND KOWARZYK[47] to the conclusion that an enzyme other than thrombin was responsible for prothrombin conversion, albeit they concluded for unapparent reasons that this enzyme was derived from prothrombin.

The properties of thrombokinase are similar to those reported for Factor X[48]. Factor X deficiency is primarily defined as a clinical bleeding state; Factor X preparations, by their ability to correct, *in vitro*, the coagulation defect in the deficient plasma. A secondary definition of Factor X is its activation with Russell viper venom. The assay system described by DENSON[15], using charcoal-adsorbed plasma and Russell viper venom has been utilized in scanning eluates from hydroxyapatite columns for Factor X. It was found that the chromatographic peak with Factor X activity was the only peak to yield active thrombokinase.

The original definition of autoprothrombin C, its method of isolation, and its assay systems provide evidence that it also may be identical with thrombokinase as prepared and measured by MILSTONE[47,49]. SPAET[50] has compared the properties of thrombokinase, autoprothrombin C, and Factor X and found no significant differences.

Since the IR-prothrombin generated thrombin, albeit slowly, in the absence of added thrombokinase, it is assumed that detectable amounts of the latter enzyme or its proenzyme were present in the preparation. However, the CNTS- and HAIR-prothrombin preparations showed greater stability, yet yielded similar results when used as substrates for thrombokinase. This observation coupled with the linear relationship seen in Fig. 2 leads to the conclusion that the necessary material for thrombin generation is found in significant amounts only in the added thrombokinase. (From the information in Fig. 2, a Michaelis constant of $7 \cdot 10^{-5}$ M has been calculated.)

This conclusion is further substantiated by the linear relationship seen in Fig. 1 where the rate of product formation is proportional to the amount of enzyme added. Moreover, these data imply that only a single component of the heterogeneous protein preparation is responsible for the conversion of the prothrombin. Since the rate of thrombin generation was constant over a wide range of thrombin concentrations (Fig. 7), autocatalysis cannot be involved as a significant factor in these experiments.

The pH optimum for the conversion in this system is in the region of 7.5. The pH optimum found by MILLER (ref. 16 and personal communication) for the polycation conversion is pH 7.0–8.5, depending on the polycation used. In most cases, however, it is somewhat higher than that found above. FERGUSON[51] has reported an acid pH optimum, but this was in a more complex prothrombin converting system.

The temperature optimum of 22° is relatively low for an enzymatic reaction. MILLER[16] has interpreted this finding as an argument that the poly-L-lysine conversion of prothrombin to thrombin is a non-enzymatic reaction. The formation of insoluble precipitates at the higher incubation temperatures may mean the formation of a more

heat-labile intermediate, since the individual components of the reaction mixture did not demonstrate this phenomenon.

The tolerance of this system to high salt concentration may be related to the finding of others that high salt concentrations activate prothrombin. It has been assumed that enzyme inhibition by high salt concentration is due to disruption of tertiary structure[20]. If such is the case thrombokinase must either be active in its disrupted state or be very difficult to disrupt. The degree of sensitivity of this reaction to Ca^{2+} is similar to that found by MILSTONE, though it is to be stressed that Ca^{2+} is not required for this reaction.

A characteristic of bovine thrombokinase is its inhibition by soybean trypsin inhibitor. The experiments above, using similar test systems, show that soybean trypsin inhibitor inhibits human prothrombin conversion by human thrombokinase.

GLENDENING AND PAGE[39] showed that soybean trypsin inhibitor inhibited the conversion of prothrombin by binding to prothrombin or an intermediate in the conversion of prothrombin to thrombin. SHULMAN AND HEARON[41] with more quantitative data noting the relationship between rate and yield, and the stoichiometric relationship between prothrombin and soybean trypsin inhibitor agreed with the view that soybean trypsin inhibitor reacted with an intermediate of prothrombin. The method used for preparing prothrombin by these investigators does not remove Factor X.

BRECKENRIDGE AND RATNOFF[42] have espoused the view that the ultimate activator of prothrombin is active Factor V and that while soybean trypsin inhibitor inhibits Factor X this step is prior to activation of Factor V. If this were the case prothrombin conversion would not be stopped once it is started other than by decay of the active Factor V. Fig. 7 shows that in the experimental situations above such is not the case.

SPAET[50] has already reported that bovine thrombokinase is not inhibited by DFP, and our findings are consistent with this. K. D. MILLER's polycation activation of prothrombin was also not inhibitable by DFP (personal communication).

TAME esterase activity was found in the human thrombokinase. While this is not an unique characteristic of thrombokinase, it is of interest to note that TAME esterase activity has been found in the Factor X of ESNOUF AND WILLIAMS[48] and the autoprothrombin C of MARCINIAK[49]. During the purification of this material the prothrombin-converting activity is associated with TAME hydrolytic activity and is separable from the clotting and TAME hydrolytic activity of thrombin. MILSTONE[21] has found this to be true in more highly purified preparations.

The studies reported here have shown that human thrombokinase will, in the absence of any other added reagent, convert prothrombin to thrombin. Soybean trypsin inhibitor completely blocks this reaction. The human enzyme, like bovine thrombokinase, seems to hydrolyze TAME, although in the preparations used there was TAME hydrolytic activity that was not blocked by soybean trypsin inhibitor. Presumably this is because of contaminating enzymes in the preparation. The effects on prothrombin conversion seen with calcium, phospholipid and adsorbed beef serum are similar to those reported by MILSTONE[6–9].

There is evidence to suggest that the products of the action of thrombokinase on prothrombin are not the results of non-specific proteolysis, but are related to the biological activation[10]. Other evidence, showing a relationship between the yield of thrombin and the rate of its formation, implies additional pertinent interactions[24,41].

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