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# Activation of Human Prothrombin by Stoichiometric Levels of Staphylocoagulase*

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**The activation of human prothrombin by the bacterial protein staphylocoagulase proceeds via the formation of a very stable equimolar complex. Unmasking of the active center in the prothrombin moiety of the complex is not caused by limited proteolysis. The kinetics of activation of human prothrombin by staphylocoagulase has been studied. The second order rate constant at pH 7.5, 37 °C, is 3.3 × $10^6$ $M^{-1}$ $s^{-1}$. This reaction rate is close to reported diffusion-controlled rates of protein-protein interaction. The dissociation constant of the complex was too low to be measurable. From the kinetic data it is assumed that the first order rate constant for dissociation is orders of magnitude less than $10^{-5}$ $s^{-1}$. However, dissociation of the complex did occur in the presence of sodium dodecyl sulfate. Equimolar amounts of staphylocoagulase protect human thrombin, but not human factor $X_a$ and bovine thrombin, against inactivation by antithrombin III. From these findings we postulate that tertiary structural changes in the thrombin region of prothrombin caused by a highly specific interaction between staphylocoagulase and that region unmask the active site.**

We have previously reported (1) that staphylocoagulase, the procoagulant exoprotein from certain strains of *Staphyloccocus aureus*, induces thrombin activity in prothrombin by virtue of the changes it brings about in the prothrombin molecule during the formation of a stoichiometric complex between the molecules. No cleavage of a peptide bond could be demonstrated in this process. Therefore, the interaction between staphylocoagulase and prothrombin is an interesting example of the production of a fully developed active site in a zymogen of the serine protease family without limited proteolysis taking place.

The interaction between streptokinase and plasminogen is another example of full expression of an active site in a zymogen as the result of protein-protein interaction. The active site in the modified plasminogen moiety intramolecularly converts plasminogen to plasmin. Thus, catalytic amounts of streptokinase are required for the activation of plasminogen into plasmin (2). It is apparent that this is not the case in the activation of human prothrombin by staphylocoagulase.

Since the original article was published, much more has become known about the protein chemistry of prothrombin (see Ref. 3 for a review) and also a much more efficient way for the purification of staphylocoagulase has been found (4). In the light of this new knowledge, we wanted to reconfirm and extend our original work (1).

We wanted to establish the following points. (*a*) What are the rate constants of the interaction between prothrombin and staphylocoagulase? (*b*) What is the location of staphylocoagulase binding sites on prothrombin? (*c*) How do the enzymatic properties of staphylocoagulase-induced activity and of normal thrombin compare? In addition, a spectrophotometric method for the determination of staphylocoagulase was developed.

## EXPERIMENTAL PROCEDURES

### *Materials*

The chromogenic substrate S2238 (D-Phe-L-Pip-L-Arg·*p*-nitroanilide) was obtained from AB Kabi-Diagnostica. p-Nitrophenyl-p-guanidinobenzoate/HCl was from Nutritional Biochemical. DFP,[1] soybean trypsin inhibitor, bovine serum albumin, ovalbumin, and *Echis carinatus* venom were obtained from Sigma. Antithrombin III was purchased from AB Kabi-Diagnostica. CNBr-activated Sepharose 4B, SP (sulfopropyl) -Sephadex C-50, Sephadex G-25, and protein standards for electrophoresis were obtained from Pharmacia. [$^3$H]DFP was obtained from Amersham. Heparin, soybean trypsin inhibititor, and bovine prothrombin were coupled to CNBr-activated Sepharose 4B following the method of Cuatrecases (5). All reagents used were of the highest grade commercially available.

### *Methods*

*Proteins*—Bovine prothrombin was prepared according to the method of Owen *et al.* (6). Before storage at −80 °C, the prothrombin preparations were passed through a column (1.0 × 20 cm) of SP-Sephadex and soybean trypsin inhibitor Sepharose 4B in order to remove small amounts of thrombin and factor $X_a$ which might be present in the preparation.

Human prothrombin was purified in a four-step procedure consisting of 1) barium citrate adsorption and elution, 2) DEAE-Sephadex gradient elution, 3) heparin-Sepharose chromatography, and 4) heparin-Sepharose gradient elution. This procedure is identical with that described for the isolation of human factor X (7), with two exceptions: 1) in all steps, benzamidine-HCl was present in a concentration of 10 mM and 2) in the last step, in which prothrombin and factor X are separated, the prothrombin- and factor X-containing pool obtained after the third step was dialyzed against 10 mM benzamidine-HCl, 10 mM triethanolamine-HCl, pH 6.35. After the addition of $CaCl_2$ (final concentration, 3 mM), the dialysis residue was applied to a heparin-Sepharose column previously equilibrated with 3 mM $CaCl_2$, 10 mM benzamidine, 10 mM triethanolamine-HCl, pH 6.35 (conductivity, 2 mS). The column was washed with 100 ml of equilibration buffer before the application of a linear gradient of 0–400 mM NaCl (2 × 225 ml) in the same buffer; the fractions were collected in a mixture containing Tris and EGTA (pH 8.0). Under these conditions, prothrombin is retarded slightly and is eluted just before the start of the gradient, whereas factor X is eluted at a conductivity of about 20 mS.

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[1] The abbreviations used are: DFP, diisopropyl fluorophosphate; DIP, diisopropyl phosphoryl; SDS, sodium dodecyl sulfate.

Fractions containing prothrombin were pooled, dialyzed against 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, and further handled as described for bovine prothrombin. The prothrombin preparation was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. Prothrombin concentration was determined after complete activation with *Echis carinatus* venom followed by active site titration with *p*-Nitrophenyl-*p*-guanidinobenzoate/HCl according to Chase and Shaw (8). Human thrombin and prethrombin 1 were generated from prothrombin and purified to homogeneity as described by Rosing *et al.* (9) and Owen *et al.* (6), respectively.

Staphylocoagulase was purified essentially as described by Igarashi *et al.* (4). A strain of *Staphylococcus aureus* (originally strain 104 of Tager (10)) was stored at −70 °C in broth. Mass culture was performed according to the method of Engels *et al.* (11). The culture of *S. aureus* was centrifuged at 6000 × *g* for 30 min at 4 °C. The culture supernatant was made 1 M in NaCl and 0.02% in Na-azide. The supernatant was applied to the bovine prothrombin-Sepharose column, equilibrated with 50 mM Tris, 1.0 M NaCl, 0.02% Na-azide, pH 7.4, at room temperature. The flow rate was maintained at 150 ml/h. The column was washed with the equilibration buffer until the $E_{280}$ of the effluent was less than 0.01.

Staphylocoagulase was eluted with 50 mM Tris, 1.0 M NaCl, 1.0 M NaSCN, and 0.02% Na-azide, pH 7.4. The staphylocoagulase containing fractions were pooled and gel-filtered on Sephadex G-25, equilibrated in 50 mM Tris, 100 mM NaCl, 0.02% Na-azide, pH 7.4.

*Electrophoresis*—Disc gel electrophoresis in the presence of SDS was performed according to Laemmli (12) and native disc gel electrophoresis was carried out by the method of Davis (13). Analytical gel electrophoresis on gradient pore slab gels was carried out with PAA 4/30 gels(Pharmacia). For native gel electrophoresis, the gels were pre-equilibrated with a recirculating buffer of 90 mM Tris, 80 mM boric acid at pH 8.4 for 1 h at 70 V. Samples were applied in 10% sucrose and electrophoresed at 150 V for 20 h.

In gel electrophoresis in the presence of SDS, the samples were heated for 2 min at 100 °C in the presence of 2% SDS, followed by an incubation for 2 h at 37 °C in the presence of 5% mercaptoethanol.

*Determination of $NH_2$-terminal Amino Acid*—The $NH_2$-terminal amino acid was determined by the method of Gray (14). The dansylamino acids were identified on polyamide sheets (15).

*Amino Acid Analysis*—For amino acid analysis, samples were prepared by the method of Moore and Stein (16) and analyzed according to the method of Dilley *et al.* (LKB, Protein Chemistry Notes No. 11), employing an LKB model 4400 amino acid analyzer.

*Protein Concentration*—Protein concentrations were obtained by absorbance measurements at 280 nm. The extinction coefficients ($E^{1\%,\,1\,cm}_{280\,nm}$) of human prothrombin, human prethrombin 1, human thrombin, and bovine prothrombin were assumed to be 13.6 (17), 17.8 (18), 16.2 (17), and 15.5 (6), respectively. The extinction coefficient of staphylocoagulase was determined by the methods of Bradford (19) and Lowry *et al.* (20) using bovine serum albumin as a standard. The molar concentration of staphylocoagulase was calculated from the molecular weight as determined by SDS-acrylamide gel electrophoresis and from the extinction coefficient.

*Determination of $K_m$ and $V_{max}$ Values of the Thrombin and Prothrombin·Staphylococagulase-catalyzed Hydrolysis of S2238*—Initial rates were measured at enzyme concentrations of $8.51 \times 10^{-2}$ nM and $5.40 \times 10^{-2}$ nM for human thrombin and prothrombin·staphylocoagulase, respectively. Substrate concentrations ranged from 2.75 to 38.5 μM. The measurements were carried out in cuvettes (thermostated at 37 °C) containing a final volume of 2.00 ml of 50 mM Tris, 100 mM NaCl, and 0.5 mg of ovalbumin/ml, pH 7.50.

The enzyme concentrations were determined by active site titration (6). Data were plotted according to Lineweaver and Burk (21). $K_m$ and $V_{max}$ were determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden (22).

*Kinetic Analysis*—The rate constant of the association between prothrombin and staphylocoagulase was determined by adding either one of the proteins in a series of varying concentrations to a fixed amount of the other in a cuvette in which the substrate S2238 was present. As substrate conversion is proportional to enzyme concentration, the acceleration of absorbance increase reflects the generation of the enzymatically active complex (See Fig. 5 for a typical recording trace).

If one of the two reactants is present in excess, the reaction will be apparently first order.

$$A + B \xrightarrow{k} E$$

$d(E)/dt = k[(A)_0 - (E)_t]$ where $k = k_a(B)$. $(A)_0$ = initial concentration of $A$, $(E)_t$ = enzyme concentration at time $t$; hence

$$(E)_t = (A)_0[1 - e^{-kt}] \quad (1)$$

The generation of $E$ is measured in a second reaction where

$$d(P)/dt = k_{cat}(E)_t \qquad (S \gg K_m) \quad (2)$$

substituting the value of $E_t$ into Equation 2 yields

$$d(P)/dt = k_{cat}(A)_0[1 - e^{-kt}] \quad (3)$$

$$(P)_t = k_{cat}(A)_0 t + [k_{cat}/k](A)_0[e^{-kt} - 1]$$

where $(P)_t$ = concentration of *p*-nitroaniline at time $t$ or

$$(P)_t = k_{cat}(A)_0[t - \tau] + [k_{cat}/k](A)_0 e^{-kt} \quad (5)$$

where $\tau = 1/k$. If $t \gg \tau$, then $e^{-kt} \rightarrow 0$ and

$$(P)_t = (Q)_t = k_{cat}(A)_0 t - [k_{cat}/k](A)_0 \quad (6)$$

$Q_t = f(t)$ is the straight line that can be drawn through the linear part of the experimental trace ($L$).

The pseudo-first order rate constant ($k$) can be determined in two ways: 1) from Equation 5 it follows that $\tau = 1/k$ can be found as the intercept of line $L$ with the abcissa (see Fig. 5) or 2) from Equations 5 and 6 it follows that

$$\ln[(P)_t - (Q)_t] = \ln[k_{cat}/k] - kt \quad (7)$$

$(P)_t - (Q)_t$ can be graphically determined from the vertical distance between the experimental trace and line $L$ (see Fig. 5). When $\ln[(P)_t - (Q)_t]$ is plotted against time, the slope of the straight line will be equal to $k$.

## RESULTS

*Spectrophotometric Assay for Staphylocoagulase Activity*—To a cuvette are added: 1.78 ml of a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 0.5 mg/ml of ovalbumin, pH 7.5, 20 μl of prothrombin solution (175 nM), and 100 μl of sample (or sample diluted with buffer). After a 10-min incubation at 37 °C, 100 μl of a 5 mM solution of S2238 is added and the absorbance change at 405 nm is recorded. The temperature is maintained at 37 °C. A blank is run without prothrombin. The amount of staphylocoagulase can be determined from comparison with the activities of solutions of active site-titrated human thrombin. As a first approximation, 1 μmol of staphylocoagulase will cause an absorbance increase of 25,000 $A$/min.

Amounts of staphylocoagulase above the prothrombin concentration cannot be measured, *i.e.* 3.0 pmol is the upper limit. The absorbance change in that case will be 0.075 $A$/min. In crude preparations, a blank activity can be observed that may be subtracted. If a preparation is contaminated with a proteolytic enzyme that activates prothrombin, a nonlinear increase in absorbance will indicate this complication.

*Purification and Partial Characterization of Staphylocoagulase*—Staphylocoagulase was purified essentially as described by Igarashi *et al.* (4). A summary of the purification procedure is given in Table I. The culture was not filtered before it was applied to the prothrombin-Sepharose 4B column as described by Igarashi *et al.* (4), because filtration through a 450-nm Millipore filter results in a 50% loss in staphylocoagulase activity and unfiltered preparations gave as good purification as filtered ones did. Under the growth condition used, 5–6 mg of staphylocoagulase per liter of culture is produced.

An electrophoretic analysis of staphylocoagulase is shown in Fig. 1. The purified staphylocoagulase is homogeneous in SDS-polyacrylamide gel electrophoresis both in the presence and absence of a reducing agent. The preparation is also homogeneous when analyzed by the method of Davis (13). Upon overloading the gels with a high amount of protein (50

TABLE I

*Purification of staphylocoagulase*

Values given are for a typical preparation.

| Purification stage | Volume | Absorbance | Concentration | Total amount | Specific activity | Yield | Purification |
|---|---|---|---|---|---|---|---|
| | *ml* | $A_{280}$ | $\mu M$ | *μmol* | $\mu M/A_{280}$ | % | *–fold* |
| Culture | 3000 | 4.9 | 0.04 | 0.12 | $8.0 \times 10^{-3}$ | 100 | 1 |
| Prothrombin-Sepharose eluate after G-25 filtration | 42 | 0.30 | 2.1 | 0.09 | 7.0 | 75 | 875 |

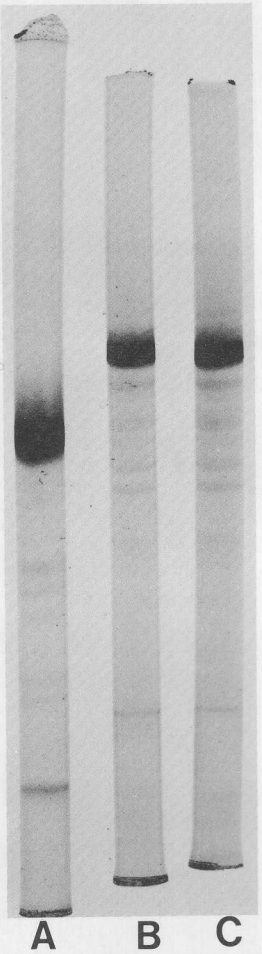


FIG. 1. **Polyacrylamide gel electrophoresis of staphylocoagulase.** *A,* native disc gel electrophoresis of staphylocoagulase, 20 μg. *B,* SDS-gel electrophoresis of staphylocoagulase, 10 μg. *C,* SDS-gel electrophoresis of reduced staphylocoagulase, 10 μg.

μg), small additional bands with a higher electrophoretic mobility could be detected. A duplicate gel, run in the system described by Davis (13), was sliced and each slice (2 mm) was soaked in a minimal amount of a Tris/NaCl buffer, pH 7.5, containing 1% ovalbumin. Staphylocoagulase activity coincided with the stained protein band. In gels overloaded with the protein, activity was also found at the positions of the faster migrating protein bands (data not shown).

The molecular weight of staphylocoagulase as determined by SDS-gel electrophoresis was found to be 54,000. The extinction coefficient ($E^{1\%,\,1\,cm}_{280\,nm}$) as determined for three preparations by the method of Bradford (19) was found to be 10.0. The extinction coefficient as estimated by the method of Lowry *et al.* (20) is 11.0. The amino acid composition of purified staphylocoagulase is shown in Table II along with data taken from the literature. Purified staphylocoagulase does not contain carbohydrate when analyzed by the procedure of Dubois *et al.* (23) for hexoses and by the method of Warren (24) for sialic acids. Aspartic acid was the only $NH_2$-terminal amino acid residue indentified.

TABLE II

*Amino acid composition of staphylocoagulase*

The mean of four different preparations is given.

| Amino acid | Strain 104 | Literature values | |
|---|---|---|---|
| | | Strain 4[a] | Strain 213[b] |
| | *g amino acid/100 g protein* | | |
| Asparagine or aspartic acid | 9.54 | 13.18 | 13.45 |
| Threonine | 8.22 | 4.74 | 6.07 |
| Serine | 5.79 | 3.71 | 3.15 |
| Glutamine or glutamic acid | 16.70 | 17.58 | 15.45 |
| Proline | 5.02 | 2.82 | 4.15 |
| Glycine | 5.55 | 3.38 | 2.0 |
| Alanine | 4.98 | 5.09 | 2.31 |
| Half-cystine | | 1.64 | |
| Valine | 6.40 | 6.96 | 5.0 |
| Methionine | 1.26 | 1.51 | 2.77 |
| Isoleucine | 4.49 | 6.42 | 4.69 |
| Leucine | 7.42 | 7.70 | 6.38 |
| Tyrosine | 4.63 | 3.99 | 8.38 |
| Phenylalanine | 2.86 | 4.53 | 3.23 |
| Tryptophan | | 0.60 | |
| Histidine | 2.02 | 1.89 | 2.61 |
| Lysine | 12.86 | 9.10 | 15.99 |
| Arginine | 3.30 | 4.01 | 2.69 |

[a] Taken from the data of Bas *et al.* (25).
[b] Taken from the data of Igarashi *et al.* (4).

*The Activation of Human Prothrombin by Staphylocoagulase*—Various amounts of human prothrombin were added to plastic tubes containing 0.22 mg of staphylocoagulase per ml of Tris/HCl buffer going from excess staphylocoagulase to excess prothrombin. The final volume of the reaction mixtures was 0.200 ml. The tubes were incubated at 37 °C for 10 min. Aliquots were withdrawn and assayed from thrombin activity. From the same reaction mixtures, samples were taken to analyze the reaction products by gradient pore gel electrophoresis. Subsequently, the reaction mixtures were incubated overnight at 4 °C in the presence of $5 \times 10^{-4}$ M [$^3$H]DFP ($1.5 \times 10^{13}$ cpm/mmol). Incorporation of [$^3$H]DIP into prothrombin was measured as described in the legend to Fig. 2.

The addition of purified human prothrombin to purified staphylocoagulase results in the generation of thrombin-like activity toward the chromogenic substrate S2238. Fig. 2 shows that maximal activity is reached when both reactants are present in equimolar concentrations. It is also shown in Fig. 2 that the generation of amidase activity coincides with the ability to incorporate [$^3$H]DIP. In the presence of a 2-fold molar excess of prothrombin to staphylocoagulase, the [$^3$H]DIP incorporation is identical with the incorporation at equimolar concentrations of both reactants. This indicates that there is no measurable exchange between the free prothrombin and prothrombin associated with staphylocoagulase in a time span of 15–20 h. Incorporation of [$^3$H]DIP occurs in parallel with the loss of amidase activity (data not shown). At maximal inhibition of the thrombin-like activity, 1.1 mol of

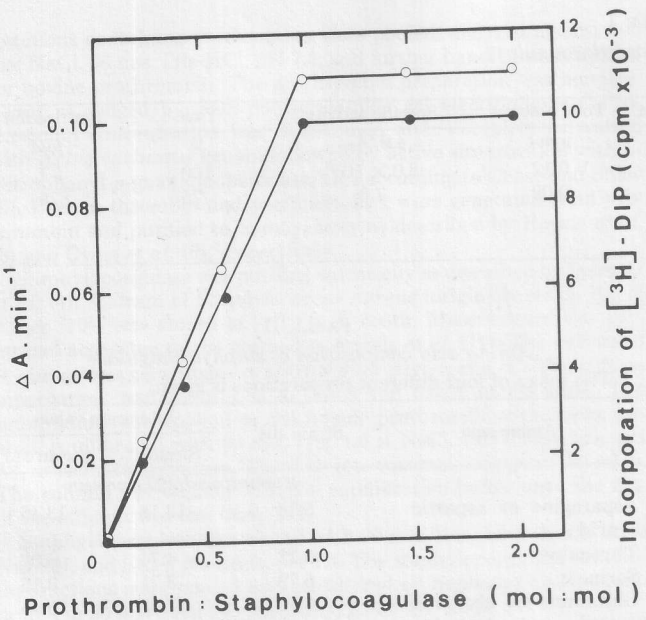


FIG. 2. **Titration of staphylocoagulase with human prothrombin.** Staphylocoagulase (3.6 μM in 50 mM Tris/HCl, 100 mM NaCl, pH 7.5) was incubated for 10 min at 37 °C with varying concentrations of prothrombin in the above buffer. The prothrombin concentration was varied from 0.7 μM to 7.0 μM. Aliquots were taken and assayed for amidase activity (*closed circles*) as described under "Experimental Procedures." From the same reaction mixtures, aliquots were taken and incubated in the presence of $5 \times 10^{-4}$ M [$^3$H] DFP overnight at 4 °C. The [$^3$H]DIP incorporation was determined by placing 20-μl aliquots on Whatman No. 3MM filter paper (2 × 2 cm), washed in cold 5% trichloroacetic acid four times. The radioactivity bound to the filter was then determined in a Packard Tri-Carb liquid scintillation counter after the addition of 10 ml of Pico-Fluor 15 (Packard). *Open circles* refer to the [$^3$H]DIP incorporation experiment.

[$^3$H]DIP was incorporated per mol of prothrombin. The prothrombin to inhibitor ratio was 1:140. More evidence for the formation of a stoichiometric complex between prothrombin and staphylocoagulase was obtained by means of gel electrophoresis.

Fig. 3*A* shows that, at a molar ratio of staphylococagulase to prothrombin of 1.0, both proteins migrate as one major protein band with an apparent molecular weight of 184,000. The presence of a minor protein band with an apparent $M_r$ = 155,000 indicates that some degradation of prothrombin and/or staphylocoagulase in the complex may occur. Because of the thrombin-like activity of the prothrombin·staphylocoagulase complex, it can be assumed that one of the degradation products is prethrombin 1. In order to identify the protein band with $M_r$ = 155,000, an equimolar mixture of prethrombin 1 and staphylocoagulase was also analyzed by

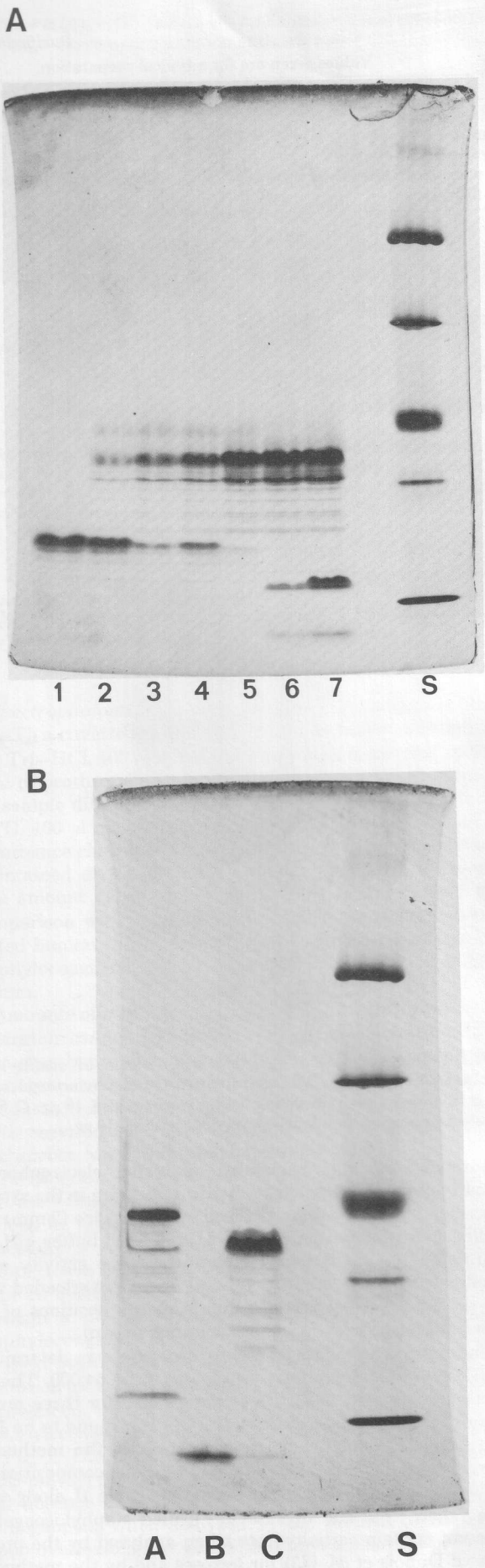


FIG. 3. **Gradient pore gel electrophoresis of prothrombin·staphylocoagulase and prethrombin 1·staphylocoagulase complex.** *A,* titration of staphylocoagulase with prothrombin. Samples (10 μl) were taken from the mixtures as described under Fig. 2. The molar ratios of prothrombin to staphylocoagulase are *1,* 0; *2,* 0.2; *3,* 0.4; *4,* 0.6; *5,* 1.0; *6,* 1.5; and *7,* 2.0. Electrophoresis was performed as described under "Experimental Procedures." Standard proteins (*S*) are in order of decreasing molecular weight: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000). *B,* formation of prethrombin 1·staphylocoagulase complex. *A,* prothrombin·staphylocoagulase complex, 5 μg. *B,* prethrombin 1, 3 μg. *C,* prethrombin 1·staphylocoagulase complex, 5 μg. *S,* standard proteins as described under *A.* Electrophoresis was carried out as described under "Experimental Procedures."

gradient pore gel electrophoresis (Fig. 3*B*). It is seen that the protein band with an apparent molecular weight of 155,000 corresponds to the staphylocoagulase·prethrombin 1 complex. In addition, it is also shown (Fig. 3*A*) that, at a molar excess of prothrombin, a protein band corresponding to prethrombin 1 ($M_r$ = ~52,000) appears in the electrophoretogram.

We investigated whether the observed degradation might result in a decrease in activity. Therefore, staphylocoagulase was titrated with prethrombin 1. The same stoichiometric binding as was demonstrated for prothrombin and staphylocoagulase was observed (data not shown). Hemker *et al.* (1) reported that no dissociation of the prothrombin·staphylocoagulase complex was observed upon SDS-gel electrophoresis. In this experiment, they were therefore unable to exclude the possibility that proteolysis may play a role in the activation of prothrombin by staphylocoagulase, but that the products do not dissociate from the enzyme. Our analysis of the prothrombin·staphylocoagulase complex by SDS-gel electrophoresis under reducing conditions showed that dissociation of the complex does occur (Fig. 4). It is clearly seen that the generation of thrombin-like activity is not accompanied by a change in the molecular weight of the prothrombin moiety of the complex.

*Kinetics of the Interaction of Prothrombin and Staphylocoagulase*—The dissociation constant of the prothrombin·staphylocoagulase complex must be extremely low. Since apparently stoichiometric binding was achieved using protein concentrations of $1.4 \times 10^{-9}$ M under conditions where 5% of a free reactant would have been detectable, the dissociation constant of the complex, $K_d$, has to be smaller than $4 \times 10^{-12}$ M (Fig. 2). Also, when an excess of prothrombin was present, no [$^3$H]DIP could be seen to incorporate in uncomplexed prothrombin after 15–20 h of incubation.

A study was done of the kinetics of activation of prothrombin over a range of staphylocoagulase concentrations from 2 to 8 nM and a prothrombin concentration of 0.17 nM. In another set of experiments, the staphylocoagulase concentration was 0.4 nM and the prothrombin concentration was varied from 1.6 nM to 7.0 nM. Fig. 5 presents a representative spectrophotometric trace, obtained by the addition of staphylocoagulase to a cuvette containing prothrombin and S2238. A detailed description of the experiment is given in the legend to this figure. The first order rate constants were obtained from these experiments by the two methods described under "Experimental Procedures." The data obtained by both methods agreed within experimental error.

The validity of back-extrapolation of the linear part of the trace (*i.e.* the activity after the formation of the complex has reached completion) was checked by the following experiment. Staphylocoagulase and prothrombin were preincubated in the absence of S2238. After a sufficient time of incubation, the amidase activity was determined. In all experiments, the activities found in this way agreed within 5% with the activities found if the formation phase of the complex was included in the measurement. The linear relationship between $\ln(P_t - Q_t)$ *versus* time (as shown in Fig. 6) indicates that the kinetics of complex formation is adequately described by the theory. The reaction constant ($k$) found was replotted against the concentration of the reactant present in excess (staphylocoagulase or prothrombin) as presented in Fig. 7, *A* and *B*, respectively. The second order rate constant ($k_a$) of the reaction between prothrombin and staphylocoagulase as determined from the replot of $k$ *versus* the concentration of the reactant in excess was $3.32 \times 10^6$ liters mol$^{-1}$ s$^{-1}$ and $3.27 \times 10^6$ liters mol$^{-1}$ s$^{-1}$ with, respectively, prothrombin and staphylocoagulase in excess.

*The Binding Sites on Prothrombin for Staphylocoagu-*

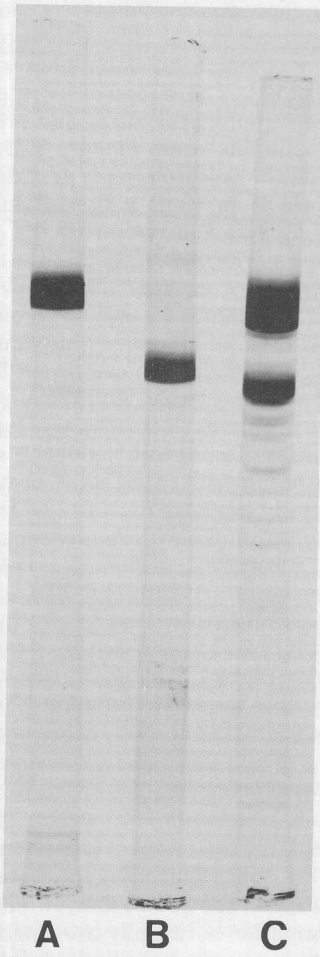


FIG. 4. **SDS-gel electrophoresis of a prothrombin·staphylocoagulase complex.** *A,* reduced prothrombin, 13 μg. *B,* reduced staphylocoagulase, 18 μg. *C,* reduced equimolar complex of prothrombin (13 μg) and staphylocoagulase (18 μg). Experimental details were as described under "Experimental Procedures."

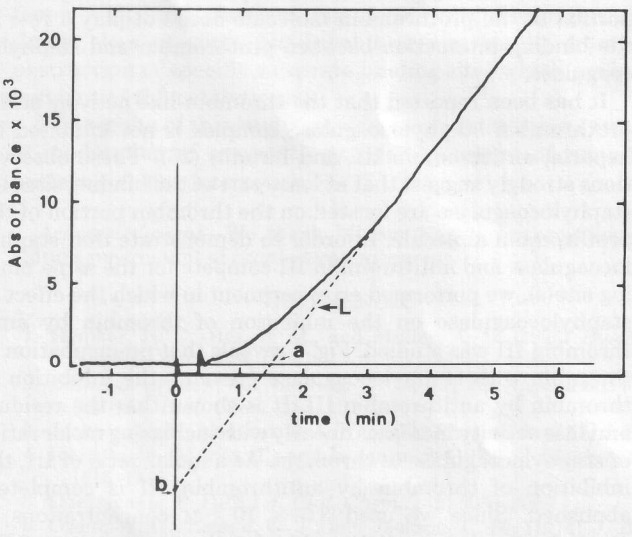


FIG. 5. **Generation of amidase activity from prothrombin by staphylocoagulase.** Amidase activity was measured continuously by adding 100 μl of prothrombin (3.5 nM) and 100 μl of S2238 (4.7 mM) to 1.75 ml of Tris buffer, pH 7.5 at 37 °C, followed by the addition of 50 μl of staphylocoagulase (80 nM) at zero time to start the reaction. The *abscissa* is drawn through the base-line obtained before the reaction started and the *ordinate* through the zero time point of the reaction. The intercept, $a$, of line $L$ on the *abscissa* is $1/k$. The intercept, $b$, on the ordinate is $-k_{cat}(A_0/k)$. See "Kinetic Analysis" for additional details.

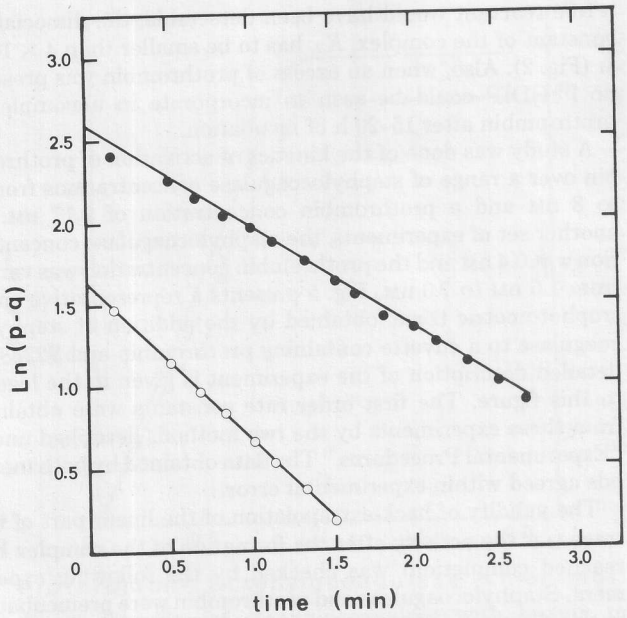


FIG. 6. **Determination of the pseudo-first order reaction constant, *k*, from a ln($P_t - Q_t$) *versus* time plot.** Staphylocoagulase, at final concentrations of 2.0 nM (●) and 8.0 nM (○), was added to cuvettes containing prothrombin (0.17 nM) and S2238 (0.24 mM). ln($P_t - Q_t$) values were graphically determined as described under "Experimental Procedures."

*lase*—In order to locate the binding sites on prothrombin for staphylocoagulase, we have studied the interaction between staphylocoagulase and prothrombin activation products, *i.e.* prethrombin 1 and thrombin. It was concluded from the experiment shown in Fig. 3*B* that staphylocoagulase not only complexed with prothrombin, but also with prethrombin 1. It appeared to do so in a 1:1 stoichiometric complex, showing the same specific activity toward S2238 as did an equimolar complex of staphylocoagulase and prothrombin. We conclude from these observations that the prothrombin fragment 1 portion of the prothrombin molecule does not play a role in the binding interaction between prothrombin and staphylocoagulase.

It has been reported that the thrombin-like activity of the prothrombin·staphylocoagulase complex is not inhibited by heparin, antithrombin III, and hirudin (26). These observations strongly suggest that at least part of the binding sites for staphylocoagulase are located on the thrombin portion of the prothrombin molecule. In order to demonstrate that staphylocoagulase and antithrombin III compete for the same binding site(s), we performed an experiment in which the effect of staphylocoagulase on the inhibition of thrombin by antithrombin III was studied. Fig. 8 reveals that preincubation of thrombin with staphylocoagulase prevents the inhibition of thrombin by antithrombin III. It is shown that the residual amidase activity increases linearly with increasing molar ratios of staphylocoagulase to thrombin. At a molar ratio of 1:1, the inhibition of thrombin by antithrombin III is completely abolished. Since we used $3.0 \times 10^{-9}$ M concentrations of staphylocoagulase and thrombin for the preparation of the equimolar complex and complex formation is achieved at very close to 1:1 molar ratios, the dissociation constant of the complex has to be orders of magnitude lower than $10^{-9}$ M. Upon preincubation of thrombin with antithrombin III, staphylocoagulase is unable to supersede the antithrombin III in the antithrombin III·thrombin complex. Our observations support the notion that the inactivation of thrombin by antithrombin III is a multistep process. That is, the primary interaction between thrombin and antithrombin III (dissociation constant around $10^{-10}$ M) is followed by the formation of an acyl bond (27). The same experiments performed with bovine thrombin and human factor $X_a$ showed that staphylocoagulase had no effect on the inhibition of human factor $X_a$ and bovine thrombin by antithrombin III. This indicates a highly specific interaction between human thrombin and staphylocoagulase. Unlike thrombin, the prothrombin·staphylocoagulase complex lacks factor V and factor VIII converting activity. However, the complexes retain the amidolytic and fibrinogen clotting activities of thrombin (Table III). Because the interaction between thrombin and staphylocoagulase results in the same obstruction of specific substrate binding sites, we concluded that staphylocoagulase interacts with portions of the thrombin molecule which are involved in the macromolecular substrate binding sites, except those for fibrinogen. It was also found that staphylocoagulase inhibits human thrombin-induced platelet activation.[2]

## DISCUSSION

In this paper, we demonstrate that the activation of prothrombin by staphylocoagulase is the result of the formation of a very stable protein-protein complex with thrombin-like activity. When a fixed amount of staphylocoagulase is titrated with prothrombin, the amidase activity and DIP incorporation increase linearly to a level which corresponds to a stoichiometric complex of prothrombin and staphylocoagulase (Fig. 2). The complex appears to be 1:1 in stoichiometry on the basis of a molar concentration for staphylocoagulase that was calculated by assuming a molecular weight of 54,000 as established by SDS-acrylamide gel electrophoresis and an extinction coefficient of 10.0. The second order rate constant for the reaction between staphylocoagulase and prothrombin as inferred from pseudo-first order kinetics is the same whether staphylocoagulase or prothrombin is present in excess. Since staphylocoagulase is in itself a nonenzymatic component, these findings support the notion of the formation of an equimolar complex. The possibility that the ultimate complex consists of a staphylocoagulase dimer ($M_r$ = 100,000) and prothrombin to make up a 2:1 ratio of molecules cannot be totally excluded (Fig. 3). However, this would seem difficult to reconcile with the extinction coefficient for staphylocoagulase determined in this study. Additionally, we wish to emphasize that the pore limit electrophoresis technique is clearly subject to the pitfall of using highly globular standard proteins for estimations of molecular weights of proteins of unknown shape. In this study, we have investigated the kinetics of the interaction of prothrombin with staphylocoagulase by using a chromogenic substrate D-Phe-L-Pip-L-Arg-*p*-nitroanilide (S2238). The generation of thrombin-like activity after addition of excess staphylocoagulase to prothrombin and vice versa, follows first order kinetics (see Fig. 6 for a representative set of first order plots). The results are consistent with a model that describes the activation of prothrombin by staphylocoagulase as a simple one-step bimolecular reaction

$$\text{Prothrombin} + \text{staphylocoagulase} \underset{k_d}{\overset{k_a}{\rightleftarrows}} \text{prothrombin} \cdot \text{staphylocoagulase}$$

The rate constant for the complex formation, $k_a$, was found to be $3.3 \times 10^6$ $M^{-1}$ $s^{-1}$. The first order rate constant for dissociation, $k_d$, was calculated to be less than $10^{-5}$ $s^{-1}$, indicating that the complex formation is virtually irreversible. Since protein-protein interactions appear to be limited to rates of about $10^6$–$10^7$ $M^{-1}$ $s^{-1}$ (28) and $k_a$ of complex formation is of the same

[2] M. J. Lindhout and E. Bevers, unpublished results.

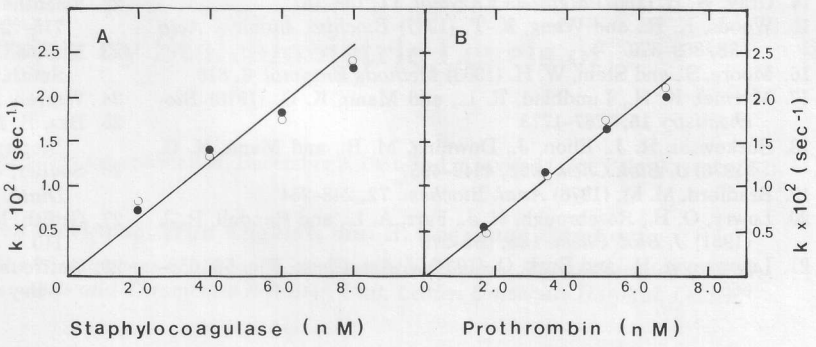


FIG. 7. **Determination of the second order rate constant for association, $k_a$, of the prothrombin·staphylocoagulase complex.** Replot of pseudo-first order reaction constants as determined from a $\ln(P_t - Q_t)$ *versus* time plot (●) and as determined from the intercept of line $L$ on the abscissa (○) *versus* the concentration of the reactant in excess. Fixed protein concentrations were: prothrombin, 0.17 nM (*A*) and staphylocoagulase, 0.40 nM (*B*). See "Kinetic Analysis" for additional details.

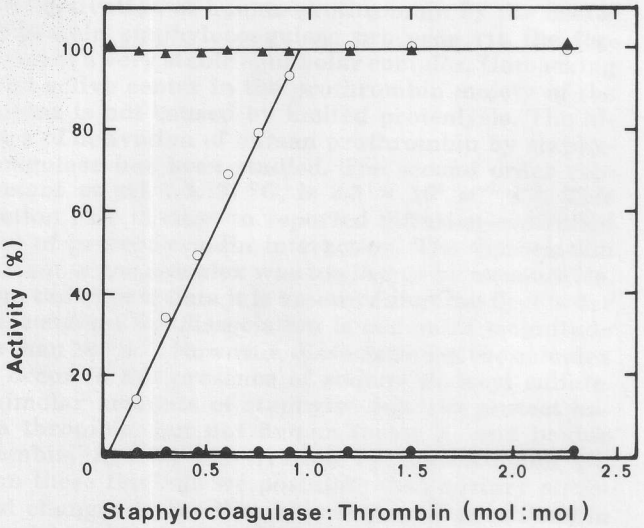


FIG. 8. **Titration of human thrombin with staphylocoagulase in the presence of antithrombin III.** Thrombin (3.0 pmol), staphylocoagulase (56 nM, 10- to 150-μl aliquots), and Tris buffer of pH 7.5 were added to a test tube containing 0.5 mg/ml of ovalbumin to a final volume of 0.95 ml and incubated for 5 min at 37 °C. Subsequently, 50 μl of antithrombin III (2.5 units/ml) was added and incubated for 10 min at 37 °C. Aliquots were taken and determined for amidase activity. The data are presented by the *open circles*. The *closed circles* depict the data of an experiment, under identical conditions, in which thrombin was incubated with antithrombin III, followed by an incubation with staphylocoagulase. The results of a titration of thrombin with staphylocoagulase in the absence of antithrombin III was depicted by *closed triangles*.

order of magnitude, it is possible that we are still dealing with a two-step process that appears as a single step. This point becomes of some importance in the discussion of mechanism of activation of prothrombin by staphylocoagulase. Peptide bond cleavages in prothrombin which occur in all other known mechanisms of prothrombin activation have not been observed in the activation of prothrombin by staphylocoagulase. The slow proteolytic degradations of the staphylocoagulase and prothrombin moieties of the complex are likely the result of the thrombin-like activity of the complex. Protein-protein interaction followed by the formation of a covalent bond is also excluded, since the complex dissociates in the presence of sodium dodecyl sulfate. Therefore, from the simplest point of view it seems reasonable that tertiary structural changes in prothrombin caused by the interaction between staphylocoagulase and prothrombin unmask the active site in the thrombin region of prothrombin. The kinetic data indicate that such a concomitant conformational change is, by itself, not rate-determining.

From the data presented in this work it might be concluded that only the thrombin region of the prothrombin molecule is involved in the interaction between staphylocoagulase and

TABLE III

*Comparison of the enzymatic activities of human thrombin (IIa), human prothrombin·staphylocoagulase (fII·SC), and human thrombin·staphylocoagulase (IIa·SC) complexes toward D-Phe-L-Pip-L-Arg-p-nitroanilide (S2238), fibrinogen (fI), factor V (fV), and factor VIII (fVIII).*

| Enzyme | S2238[a] | | Macromolecules | | |
|---|---|---|---|---|---|
| | $K_m$ | $k_{cat}$ | fI | fV | fVIII |
| | μM | $s^{-1}$ | *relative rates*[b] | | |
| IIa | 2.67 | 78 | 100 | 100 | 100 |
| fII·SC | 2.74 | 77 | 85 | <1 | <1 |
| IIa·SC | 2.60 | 69 | 90 | <1 | <1 |

[a] See "Experimental Procedures" for additional details.
[b] Rates were determined as arbitrary units of substrate conversions/min/mol of enzyme.

prothrombin. By virtue of not being inhibited by antithrombin III in the presence of staphylocoagulase, it could be shown that human thrombin binds tightly to staphylocoagulase (Fig. 8). The interaction between human thrombin and staphylocoagulase is of high specificity, since staphylocoagulase did not protect bovine thrombin and human factor $X_a$ against inactivation by antithrombin III. It is interesting to see that association of human thrombin with staphylocoagulase results in the loss of biological activity of thrombin toward factor V, factor VIII, and platelets, but not toward fibrinogen. Evidently, loss of some biological activities results from the obstruction of specific substrate binding sites which are independent of the catalytic site.

The results of this study provide a very interesting example of modulation of enzymatic activity by means of zymogen-protein cofactor interaction.

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