J. Biochem. 97, 1073–1078 (1985)

Difference in Enzymatic Properties Between α-Thrombin-Staphylocoagulase Complex and Free α-Thrombin[1]

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Received for publication, October 23, 1984

The steady-state kinetic parameters of human α-thrombin and the α-thrombin-staphylocoagulase complex as to the chromogenic substrate, H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), were determined. At pH 8.0 and 37°C, the K_m values for α-thrombin and the complex for S-2238 were 7.9 μM and 7.7 μM, respectively. The k_{cat} of this amidase reaction catalyzed by the complex was 127 s^{-1}, which had apparently decreased from the k_{cat} of 197 s^{-1} determined for free α-thrombin. This difference in the kinetic parameter between α-thrombin and the complex was also observed using the fluorogenic substrate, Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide. Moreover, the fibrinogen clotting activity of the α-thrombin-staphylocoagulase complex was less than half that of α-thrombin, suggesting that the α-thrombin active site in the complex is different in catalytic ability from that of free α-thrombin. Other evidence supporting this view was as follows: (1) The α-thrombin-staphylocoagulase complex is insensitive to antithrombin III, (2) the complex shows much weaker binding to hirudin, as compared to free α-thrombin, and (3) the amidase pH-profiles of the complex and free α-thrombin differ from each other. These results indicate that the microenvironment of the active site of α-thrombin is significantly altered by the complex formation with staphylocoagulase.

Staphylocoagulase forms a complex with human prothrombin, in a molar ratio of 1 : 1 (*1*, *2*), and the staphylocoagulase-zymogen complex, called staphylothrombin (*3*), displays the ability to clot fibrinogen and to hydrolyze the chromogenic and fluorogenic tripeptide substrates for α-thrombin (*2*, *4–6*). The equilibrium dissociation constant of this complex has been determined to be approxi-

[1] This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Abbreviations: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; Boc, *t*-butoxycarbonyl; MCA, 4-methylcoumaryl-7-amide; pNA, *p*-nitroanilide; BSA, bovine serum albumin.

mately 10^{-10} M (*7*), which is comparable with that of the α-thrombin-thrombomodulin complex ($K_d = 10^{-10}$ M) (*8*). It is known that staphylothrombin and α-thrombin differ from each other in their substrate specificities toward protein substrates and synthetic tripeptide substrates, in addition to their susceptibilities to protease inhibitors (*6*). This indicates that the functional and structural properties of the active site of staphylothrombin differ essentially from those of α-thrombin. Because free α-thrombin also forms a complex with staphylocoagulase (*9*), it is of interest to compare the kinetic properties of α-thrombin with those of the enzyme bound to staphylocoagulase. The results of such studies are presented in this paper.

MATERIALS AND METHODS

Materials—Staphylocoagulase from the culture filtrate of *Staphylococcus aureus*, strain st-213, was purified as described previously (*10*). The preparation used here contained the major species with a molecular weight of 64,000 (*11*). Human prothrombin was prepared by the method of Miletich *et al.* (*14*). Antithrombin III was isolated from bovine plasma on a heparin-Sepharose CL-6B column (*12*) prepared by the method of March *et al.* (*13*). Hirudin (1,800 units/mg), *Echis carinatus* venom and *p*-nitrophenyl *p'*-guanidinobenzoate-HCl (NPGB) were purchased from Sigma Chemical Co., St. Louis. NIH standard thrombin, Lot B-3, was a generous gift from Dr. D.L. Aronson, Division of Biologics Standards, Food and Drug Administration, Bethesda. Sodium heparin (152 I.U./mg) was obtained from Nakarai Chemicals Ltd., Kyoto. Bovine fibrinogen (type 2) was a product of Daiichi Pure Chemicals Co., Ltd., Tokyo. The fluorogenic substrate, Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide, was a product of the Protein Research Foundation, Minoh, Osaka. Chromogenic substrate S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide, was obtained from AB KABI Diagnostica, Sweden. All other chemicals were of analytical reagent grade or the highest quality commercially available.

Preparation of Human α-Thrombin—Human α-thrombin was prepared by incubating prothrombin (267 mg/184 ml) with crude *Echis carinatus* venom (8.9 mg/3 ml) at 37°C for 1 h in 0.05 M Tris-HCl, 0.1 M NaCl, pH 8.0. The mixture was dialyzed against 0.05 M sodium phosphate buffer, pH 6.5, and applied to a CM-Sepharose CL-6B column (4.5 × 12.5 cm), equilibrated with the same buffer, to remove prothrombin fragment 1 and fragment 2. The column was then washed with the equilibration buffer, and α-thrombin with clotting activity (2,520 NIH units/mg) was eluted with a linear gradient formed from 0 to 0.6 M NaCl in the same buffer at a flow rate of 60 ml per h. The yield of the purified α-thrombin was 95.6 mg and the preparation gave a single band on sodium dodecylsulfate-polyacrylamide gel (8%) electrophoresis under the unreduced conditions (*25*).

Determination of Clotting Activity—One percent bovine fibrinogen (98% clottable protein) in 0.3 M NaCl was diluted to obtain a 0.3% solution with 0.1 M Tris-HCl buffer, pH 7.2, containing 0.06 M NaCl. The clotting activity was measured by adding 20 μl of a test solution to 280 μl of 0.3% fibrinogen, which had been preincubated at 37°C for 3 min. The time for clot formation was recorded with a Fibrometer (Becton-Dickinson). The clotting activity, expressed in NIH units, was calculted by interpolating the observed clotting time on a linear log-log plot of NIH units *versus* clotting time.

Determination of Amidase Activity—A fluorogenic substrate assay using Boc-Val-Pro-Arg-MCA was performed in 0.6 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.1 mg/ml BSA. Ten μl of 5 mM peptidyl-MCA dissolved in water was added to a cuvette thermostatted at 37°C, and then 20 μl of the test sample was added and the liberated 7-amino-4-methylcoumarin was measured with excitation at 380 nm and emission at 440 nm with a Hitachi 650-10M spectrofluorometer (*15*). The amidase activity was also measured using S-2238 as follows; a mixture of 1.9 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.1 mg/ml BSA, and 100 μl of 2 mM substrate solution was preincubated at 37°C. Then, 20 μl of the test sample was added and the absorbance change at 405 nm was measured with a Hitachi model 220 spectrophotometer. The test samples were usually diluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.1 mg/ml BSA. The initial rates for determination of K_m and k_{cat} values were measured at the enzyme concentration of 1.4×10^{-10} M. Substrate concentrations ranged from 2.2 to 22 μM for S-2238 and

from 7.0 to 70 μM for Boc-Val-Pro-Arg-MCA.

Active Site Titration—The active site titration was carried out with NPGB, according to the method of Chase and Shaw (*16*).

Extinction Coefficients and Molecular Weights for Proteins—Protein concentrations were calculated from the molecular weights and absorbance ($E^{1\,cm}_{1\%}$) at 280 nm using the following data: 37,000 (*18*) and 18.3 (*17*) for human α-thrombin, and 56,000 (*19*) and 6.0 (*19*) for bovine antithrombin III. The protein concentration for staphylocoagulase was calculated from the amino acid mass/A_{280}.

RESULTS

Enzymatic Properties of α-Thrombin-Staphylocoagulase Complex—To α-thrombin at a constant concentration, various concentrations of staphylocoagulase were added and the mixtures stood at 0°C for 10 min. Then, the amidase and clotting activities of the mixture were measured using S-2238 and fibrinogen as substrates. In Fig. 1, we plotted these activities *versus* the molar ratio of staphylocoagulase to α-thrombin, taking the α-thrombin activity in the absence of staphylocoagulase as 100. By increasing the concentration of staphylocoagulase, both the amidase and clotting activities of α-thrombin were reduced, respectively, to 70% and 40%, and the reducing curves were refracted at the molar ratio of staphylocoagulase to α-thrombin of 1 : 1, indicating stoichiometric complex formation of staphylocoagulase with α-thrombin.

Table I shows the steady-state kinetic parameters of α-thrombin and the α-thrombin-staphylocoagulase complex toward the chromogenic substrate, H-D-Phe-Pip-Arg-pNA (S-2238), and the fluorogenic substrate, Boc-Val-Pro-Arg-MCA. The active site concentration of the complex titrated by means of the NPGB burst assay of Chase and Shaw (*16*) was the same as that of α-thrombin, and their acylation rates were comparable, indicating that 1 mol of the active site per mol of the complex is retained (data not shown). At pH 8.0 and 37°C, the K_m values of α-thrombin and the complex for S-2238 were 7.9 μM and 7.7 μM, respectively. The k_{cat} of the amidase reaction catalyzed by the complex was 127 s^{-1}, which had apparently decreased from the k_{cat} of 197 s^{-1} determined when free α-thrombin was the enzyme. This difference of the kinetic parameter between α-thrombin and the complex was also observed using Boc-Val-Pro-Arg-MCA as a substrate, as shown in Table I.

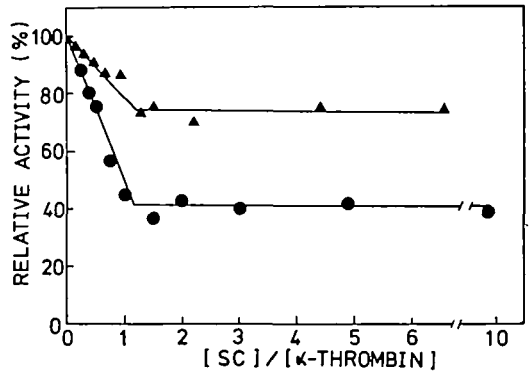

Fig. 1. Effects of staphylocoagulase (SC) on the amidase and clotting activities of human α-thrombin. α-Thrombin (1.8 μg/ml) was mixed with various concentrations of staphylocoagulase followed by preincubation at 0°C for 10 min. Then, 0.3% fibrinogen solution was added and the time for clot formation (●) was measured at 37°C. For assaying of the amidase activity, S-2238 was used as a substrate (▲) as described under " MATERIALS AND METHODS."

TABLE I. Enzymatic properties of the α-thrombin-staphylocoagulase (SC) complex and α-thrombin. Clotting activity of the α-thrombin-staphylocoagulase complex was calculated from the data in Fig. 1. K_m and k_{cat} values of α-thrombin and the complex were determined as described under " MATERIALS AND METHODS." Data were analyzed by Lineweaver-Burk plotting.

	Active site titration (%)	Clotting activity (NIH units/nmol)	S-2238			Boc-Val-Pro-Arg-MCA		
			K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
α-Thrombin	95±3	97	7.9	197	25	21	105	5.0
α-Thrombin-SC	96±3	37	7.7	127	17	25	89	3.6

On the other hand, the fibrinogen clotting activity of the complex was less than half that of free α-thrombin, suggesting that the catalytic rate of α-thrombin bound to staphylocoagulase is significantly reduced when a large protein substrate is employed.

Figure 2 shows the pH profiles of human α-thrombin and the α-thrombin-staphylocoagulase complex obtained on using Boc-Val-Pro-Arg-MCA. As expected, α-thrombin showed a bell-shaped profile with an optimum at pH 8.5. However, the pH profile of the complex differed appreciably from that of α-thrombin especially at alkaline pH, it indicated that the activity was rapidly dropped at pH 10.5. The pH stability of the α-thrombin-staphylocoagulase complex is shown in Fig. 3. The complex as well as free α-thrombin was stable in the pH range of 5.2 to 10.3 and was unstable below pH 5 and above pH 10.5. Thus, the rapid decrease in amidase activity of the complex at higher alkaline pH appeared to be due to denaturation of α-thrombin or dissociation of the α-thrombin staphylocoagulase complex.

Effects of Antithrombin III and Hirudin on the Amidase Activity of α-Thrombin-Staphylocoagulase Complex—The effects of antithrombin III and hirudin on the amidase activity of the complex were examined using Boc-Val-Pro-Arg-MCA. No inhibitory effect of antithrombin III plus heparin

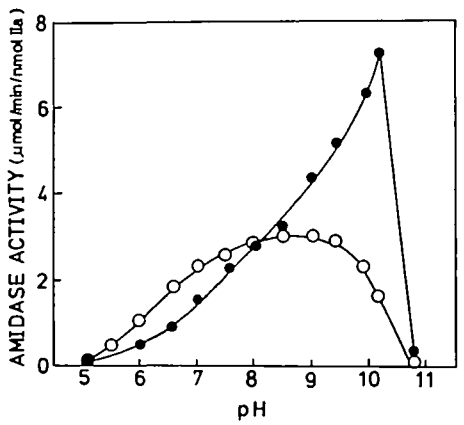

Fig. 2. Effect of pH on the amidase activity of the α-thrombin-staphylocoagulase complex and α-thrombin. The mixture containing an equal volume of α-thrombin (1.8 μg/ml) and staphylocoagulase (100 μg/ml) was preincubated at 0°C for 10 min. Free α-thrombin (1.8 μg/ml) was treated similarly. Then, 20 μl aliquots were taken and added to 600 μl of Britton-Robinson's buffer (*24*). The amidase activities of the complex (●) and free α-thrombin (○) were measured using Boc-Val-Pro-Arg-MCA.

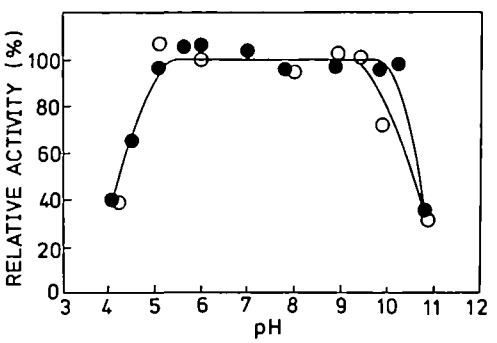

Fig. 3. Effect of pH on stability of the α-thrombin-staphylocoagulase complex and α-thrombin. α-Thrombin (0.15 mg/ml) and staphylocoagulase (0.3 mg/ml) were preincubated at 0°C for 10 min. Ten μl aliquots were taken and added to 500 μl of Britton-Robinson's buffer (from pH 4.1 to pH 10.8) followed by incubation at 37°C for 3 min. Free α-thrombin (0.3 mg/ml) was treated similarly. Then, 20 μl aliquots were taken and the amidase activities were measured at pH 8.0 as described under "MATERIALS AND METHODS." α-Thrombin-staphylocoagulase complex (●), α-thrombin (○).

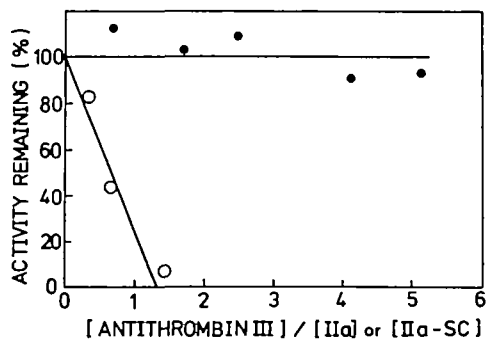

Fig. 4. Effect of antithrombin III-heparin on the amidase activity of the α-thrombin (IIa)-staphylocoagulase (SC) complex and α-thrombin. The mixture containing an equal volume of α-thrombin (6.2 μg/ml) and staphylocoagulase (14 μg/ml) was preincubated at 0°C for 10 min. Free α-thrombin (6.2 μg/ml) was treated similarly. To the mixture, various concentrations of antithrombin III-heparin were added followed by incubation at 37°C for 5 min. The residual activities of the complex (●) and α-thrombin (○) were measured using Boc-Val-Pro-Arg-MCA.

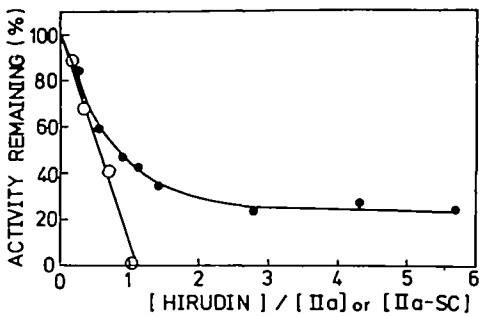

Fig. 5. Effect of hirudin on the amidase activity of the α-thrombin (IIa)-staphylocoagulase (SC) complex and α-thrombin. The conditions used were the same as those in Fig. 4. The residual amidase activities of the complex (●) and α-thrombin (○) were measured using Boc-Val-Pro-Arg-MCA.

on the complex activity was seen, even in the presence of a five-fold molar excess of antithrombin III (Fig. 4). In contrast, α-thrombin used as the control was completely inhibited as expected. Hirudin also inhibited α-thrombin but showed incomplete inhibition of the α-thrombin-staphylocoagulase complex, as shown in Fig. 5.

DISCUSSION

The results presented in this paper demonstrate that human α-thrombin bound to staphylocoagulase is altered as to its catalytic abilities toward typical substrates and protease inhibitors for α-thrombin. The evidence is as follows: (1) The catalytic rate of the complex is more significantly reduced when a large protein substrate, fibrinogen, is employed. The complex possesses 40% of the activity of free α-thrombin toward fibrinogen. (2) With regard to the tripeptide substrates, S-2238 and Boc-Val-Pro-Arg-MCA, the lower enzymatic activity shown by the α-thrombin-staphylocoagulase complex is a result of a decrease in the k_{cat} values, that are approximately 66–72% of that of α-thrombin. (3) The pH profile of the α-thrombin-staphylocoagulase complex differs remarkably from that of α-thrombin. Therefore, differences in these enzymatic properties between the complex and free α-thrombin suggest that the microenvironments of their active sites are not identical.

Antithrombin III plus heparin has no ability to inhibit the activity of the α-thrombin-staphylocoagulase complex. Possibly, complex formation blocks access to inhibitor binding sites located near the active site of α-thrombin due to steric hindrance. It is of interest that hirudin inhibits the amidase activity of the complex, although it has no inhibitory effect on either the amidase or clotting activity of staphylothrombin (*9*). This result suggests that hirudin may displace the staphylocoagulase portion from the α-thrombin-staphylocoagulase complex, resulting in the gradual loss of the amidase activity due to free α-thrombin. In fact, hirudin is known to be one of the most potent thrombin-specific inhibitors, with a K_i of 0.8×10^{-10} M (*20*), which is comparable with the dissociation constant of staphylothrombin (4.6×10^{-10} M) (*7*).

It is known that the thrombin-catalyzed activation of protein C is remarkably accelerated when thrombin is bound to thrombomodulin, which is a protein cofactor required for the activation (*21*). The thrombomodulin-thrombin complex is also altered as to its catalytic abilities toward various substrates (*22*) and platelet activation (*23*), like the α-thrombin-staphylocoagulase complex described here. However, detailed kinetic analyses of the complex have not been extensively performed. Therefore, we feel that the α-thrombin-staphylocoagulase complex, in addition to staphylothrombin (*1*, *2*), is a good model for understanding the modulation of the protease function through protein-protein interaction.

We thank Miss Mizumo Akiyoshi for her expert secretarial assistance.

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