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Kinetics of Human Thrombin Inhibition by Two Novel Peptide Inhibitors (Hirunorm IV and Hirunorm V)

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ABSTRACT. A study on the kinetics of human thrombin inhibition by two novel synthetic peptides (Hirunorm IV and Hirunorm V) and a comparison with recombinant hirudin and a commonly used thrombin inhibitor, Hirulog-1, are reported. The dissociation constants for Hirunorm IV and Hirunorm V were determined by varying the concentration of inhibitors at fixed concentrations of the chromogenic substrate Chromozym-TH (N-tosylglycyl-L-prolyl-L-arginine 4-nitroanilide acetate). Both inhibitors behaved as reversible tight-binding inhibitors of amidolytic thrombin activity. The apparent dissociation constants determined showed a linear dependence on the concentration of substrate; this finding, which indicates that the inhibition was competitive, made possible the estimation of the dissociation constants (K_I) for Hirunorm IV and Hirunorm V, which were 0.134 ± 0.014 nM and 0.245 ± 0.016 nM, respectively. Similar dissociation constants were also obtained for the two inhibitors when thrombin activity was measured with fibrinogen in the clotting assay. When tested for resistance to thrombin proteolytic activity, both inhibitors were inviolate to cleavage by thrombin. The data obtained demonstrate that both Hirunorm IV and Hirunorm V are potent and stable inhibitors of human thrombin activity. BIOCHEM PHARMACOL 52;8:1141–1146, 1996.

KEY WORDS. thrombin; thrombin inhibition; hirudin; coagulation; Hirulog; Hirunorm

Thrombin (E.C. 3.4.4.13), a serine protease, plays a major role in the control of blood fluidity as well as a number of inflammatory and proliferative processes [1]. In particular, it is critically involved in the final steps of the blood coagulation cascade, where it specifically catalyzes the conversion of fibrinogen to fibrin through the cleavage of four arginyl-glycyl peptide bonds [2]. The interaction between thrombin and fibrinogen is highly specific: fibrinogen binds to the active site and to the so-called fibrinogen recognition site, a structural domain distinct from the catalytic pocket and characterized by a well-defined cluster of positively charged residues [3–5]. Thrombin inhibition would provide an effective means of controlling thrombosis. Heparin increases the rate of thrombin inactivation by antithrombin III [6] and, for this reason, has been widely used as an anticoagulant. However, heparin does have several disadvantages, such as the necessity of frequent monitoring due to unpredictable pharmacokinetics and ineffectiveness in patients with antithrombin III deficiencies. Because of these disadvantages, many attempts have been made to find direct-acting specific and efficient inhibitors of thrombin activity. The most potent natural inhibitor of thrombin known is hirudin, a peptide isolated from the salivary gland of the leech *Hirudo medicinalis* [7]. Hirudin forms a noncovalent stoichiometric complex with human thrombin with a dissociation constant of approximately 20 fM for the natural form and 200 fM for the recombinant protein, which is not sulphated on Tyr^{63} [8]. In recent years, a considerable effort has been made to design specific thrombin inhibitors based on the structure of hirudin [9–12].

Hirunorm IV (H-Chg‖-Arg-2Nal-Thr-Asp-(D-Ala)-Gly-βAla-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr-Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-(D-Glu)-OH) and Hirunorm V (H-Chg-Val-2Nal-Thr-Asp-(D-Ala)-Gly-βAla-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr-Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-(D-Glu)-OH) have recently been proposed as inhibitory peptides closely related to hirudin. They are designed to interact through their N-terminal end with the thrombin active site in a nonsubstrate mode, while specifically binding to the fibrinogen recognition exosite. The

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‖ *Abbreviations:* Aib, α-aminoisobutyric acid; β-Ala, β-alanine; Cha, ciclohexylalanine; Chg, cyclohexylglycine; 2Nal, β-2-naphthylalanine; hPhe, omophenylalanine.

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peculiar interaction mode with the catalytic site, proper to hirudin, but not to other peptide analogues, was the result of the appropriate design of the spacer arm between the two binding moieties of the molecules.* In the present work, a study on the kinetics of human thrombin inhibition by these two novel synthetic peptides and a comparison with recombinant hirudin and Hirulog-1, the first synthetic analogue to undergo advanced clinical evaluation [13], are reported.

MATERIALS AND METHODS

Materials

The chromogenic substrate Chromozym-TH (N-tosylglycyl-L-prolyl-L-arginine (Chimica, Milan) 4-nitroanilide acetate) (>99% pure) was from Fluka. Highly purified human α-thrombin (3700 units mg^{-1} protein), human fibrinogen and *p*-nitrophenyl-*p'*-guanidinobenzoate were purchased from Sigma (St. Louis, MO). Active site titration of thrombin was performed by the method of Chase and Shaw [14] using *p*-nitrophenyl-*p'*-guanidinobenzoate as titrant. Thrombin concentration was determined using an extinction coefficient E_{280} = 1.83 $mL \cdot mg^{-1} \cdot cm^{-1}$ and a molecular weight of 36,500 Da [15]. Hirunorm IV and Hirunorm V were from Primm S.r.l., Milan, Italy, and were >98% pure. Highly purified recombinant hirudin rBKHV (>99% pure) was from Rhein Biotech GmbH (Dusseldorf, Germany). The concentration of r-hirudin was determined from calibration curves obtained by reporting enzyme activity as a function of known concentrations of thrombin: At nanomolar concentrations of thrombin, the reaction between thrombin and hirudin is stoichiometric, and the concentration of hirudin can be determined from the decrease in activity of a known concentration of thrombin. All other chemicals were of the highest purity available.

Amidolytic Assay of Thrombin Activity

Thrombin-catalyzed hydrolysis of Chromozym-TH was followed by monitoring on a Beckman DU-7 spectrophotometer the increase in absorbance at 405 nm that resulted from the release of *p*-nitroaniline. The assay was performed in polystyrene cuvettes at 25°C in 0.01 M Hepes/0.01 M Tris buffer, pH 7.4, containing, in a final volume of 0.5 mL, 0.1 M NaCl and 0.1% PEG6000 at a thrombin concentration of 0.75 nM, unless otherwise stated. The assay was started by addition of the substrate to a preincubated solution of the enzyme in the assay mixture. The amount of product formed was calculated using an extinction coefficient of 9920 M^{-1} cm^{-1} at 405 nm for *p*-nitroaniline [16]. In the inhibition studies, the addition of variable amounts of inhibitors to the reaction mixtures always preceded the addition of the chromogenic substrate.

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Fibrinogen Clotting Assay

The assay was performed at 37°C in polystyrene cuvettes in 0.01 M Hepes/0.01 M Tris buffer, pH 7.4, containing, in a final volume of 0.5 mL, 0.1 M NaCl, 0.01 M $CaCl_2$ and 0.1% PEG6000 at a thrombin concentration of 3.75 nM. The reactions were started by addition of fibrinogen (0.3 mg mL^{-1}) to the assay mixture preincubated at 37°C. The reaction was followed at 405 nm by a Beckman DU-7 spectrophotometer and clot formation assessed by monitoring the sudden increase in absorbance. Enzyme activity was quantified as previously described [9] by measuring the time required for clot formation. In the inhibition studies, variable concentrations of inhibitors were added to the reaction mixture prior to the addition of fibrinogen.

Data Analysis

To determine the apparent dissociation constants (K_I') of the tested thrombin inhibitors, steady-state velocity measurements were performed by varying the concentration of the inhibitors at different fixed concentrations of Chromozym-TH (13.4–100 μM). Because all tested inhibitors exerted their effect on the thrombin-catalyzed reaction at a concentration comparable to that of thrombin (tight-binding inhibitors), it was necessary to make an allowance for the depletion in the concentration of free inhibitor [17, 18]. Therefore, the steady-state reaction rates (v_s) measured at each inhibitor total concentration (I_t) were fitted by weighted nonlinear regression to the equation:

$$v_s = (v_o/2E_t)\{[(K_I' + I_t - E_t)^2 + 4K_I'E_t]^{1/2} - (K_I' + I_t - E_t)\} \quad (1)$$

in which v_o is the velocity observed in the absence of inhibitor, E_t is the total enzyme concentration and K_I' is an apparent inhibition constant. In the case of competitive inhibition, the true dissociation constant of the inhibitor, K_I, is related to K_I' by equation (2):

$$K_I' = K_I(1 + [S]/K_m) \quad (2)$$

where [S] is the concentration of substrate and K_m the Michaelis constant.

Even though the clotting assay measures an endpoint, the logarithm of clotting times is linearly related to the logarithm of the concentration of the enzyme in NIH clotting units [2]. From this relationship, the ratio of the inhibited thrombin activity in clotting units to the control activity of thrombin alone is equal to the velocity ratio v_s/v_o. The values of K_I' for inhibitors were determined by Eqn (1) and (2) by assuming that fibrinogen Aα cleavage by thrombin is rate-limiting [19] and that the K_m for this cleavage is 7.2 μM [20]. When the steady-state of the thrombin-catalyzed reaction was slowly achieved (as in the case of hirudin at higher ionic strength; see Results and Discussion), the progress curves of product formation were

analyzed according to the following Eqn [21, 22], which applies to slow interacting tight-binding inhibitors:

$$P = v_s t + \frac{(1-\gamma)(v_o - v_s)}{k\gamma} \ln\left(\frac{1-\gamma e^{-kt}}{1-\gamma}\right) \quad (3)$$

where: P is the amount of product formed at time t; k is an apparent pseudo-first-order rate constant which is a function of E_t, I_t, K_I' and the apparent second-order rate constant (k_I') for the reaction between the enzyme and the inhibitor, and γ is an adimensional factor related to E_t, I_t, and K_I'; k and γ are given by the expressions:

$$k = k_1' [(K_I' + E_t + I_t)^2 - 4E_t I_t]^{1/2} \text{ and}$$

$$\gamma = \frac{K_I' + E_t + I_t - [(K_I' + E_t + I_t)^2 - 4E_t I_t]^{1/2}}{K_I' + E_t + I_t + [(K_I' + E_t + I_t)^2 - 4E_t I_t]^{1/2}}$$

In the case of competitive inhibition, k_I' is related to the second-order rate constant (k_I) for the reaction between the enzyme and the inhibitor by:

$$k_I' = k_I[K_m/([S] + K_m)] \quad (4)$$

Stability of Thrombin Inhibitors to Thrombin Proteolytic Activity

Human thrombin at 1.58 nM was incubated at 25°C with 10, 20, and 40 nM inhibitor in 0.01 M Hepes/0.01 M Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.1% PEG6000. At different times, aliquots of 250 μL were withdrawn from the incubation mixtures and assayed for thrombin amidolytic activity. Samples incubated in the absence of inhibitor were used as controls.

Other Methods

Gel electrophoresis in the presence of SDS used to assess the purity of thrombin was performed according to the method of Laemmli [23], using 0.75-mm thick slab gels and 18% acrylamide. Gels were stained with silver nitrate according to the method of Wray *et al.* [24].

RESULTS AND DISCUSSION

Kinetics of human thrombin inhibition by Hirunorm IV and Hirunorm V were studied in comparison with the kinetics of inhibition by recombinant hirudin and Hirulog-1, a synthetic peptide that was reported to be a potent inhibitor of thrombin [10, 11]. Studies were performed by incubating human thrombin with several concentrations of the inhibitors at different fixed concentrations of chromogenic substrate Chromozym-TH. When tested for purity and enzyme activity, the highly purified thrombin used in the study showed a single band on SDS polyacrylamide gel electrophoresis and was 93% active as judged by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (data not shown). All peptides tested inhibited thrombin activity at concentrations comparable to that of thrombin in the incubation mixture. Thus, the data obtained were analyzed on the basis of tight-binding inhibition theory [22]. To determine whether both Hirunorm IV and Hirunorm V act as fast or slow inhibitors [25], progress curves were obtained either by starting the reaction with the enzyme or, alternatively, by adding the substrate to thrombin preincubated for 15 min with the inhibitor. Because, in both experiments, product formation increased as a linear function of time and both experiments yielded the same rate of product formation (data not shown), it can be concluded that Hirunorm IV and Hirunorm V act as fast tight-binding inhibitors. A similar result was also obtained for Hirulog-1. In the case of *r*-hirudin, the linearity of progress curves obtained by starting the reaction with the enzyme, under standard assay conditions, is indicative that steady-state velocities were reached upon mixing. This apparent fast inhibitory action of *r*-hirudin is not in contradiction with the fact that hirudin and *r*-hirudin are generally considered to act as slow tight-binding inhibitors [8, 26]. In fact, as pointed out by Stone and Hofsteenge [8], hirudin and *r*-hirudin can be classified as slow tight-binding inhibitors of thrombin only at high ionic strength. Indeed, progress curves obtained by starting the reaction with the enzyme after increasing NaCl concentration in the assay mixture from 0.1 to 0.4 M showed exponential and linear phases (Fig. 1, panel A). In contrast, the increase in NaCl concentration had no effect on the progress curves obtained with Hirunorm IV and Hirunorm V, which again behave as fast tight-binding inhibitors (Fig. 1, panel B). A decreased rate of formation of the thrombin-hirudin complex has been shown to be the cause of the slow tight-binding inhibition observed at high salt concentration [8]. The absence of exponential phases in the progress curves obtained with Hirunorm IV and Hirunorm V at high NaCl concentrations does not allow the estimation of kinetic parameters for the Hirunorm binding process, making it difficult to propose an explanation for the insensitivity of Hirunorm peptides to high ionic strength. However, the relatively high concentration of Hirunorm IV and V required, for thermodynamic reasons, to inhibit thrombin (5- to 200-fold higher than *r*-hirudin concentrations) may well play a role in favoring the binding step of thrombin-inhibitor complex formation.

Apparent dissociation constants for Hirunorm IV and Hirunorm V were determined through Eqn (1) by weighted nonlinear regression analysis of relative residual activity vs inhibitor concentration data. Typical results are shown in Fig. 2. As pointed out by Williams and Morrison [22], the calculation of the true dissociation constant, K_I, for an enzyme-inhibitor complex from the apparent dissociation constants requires, in addition to the availability of the kinetic parameters associated with the enzymatic reaction, the knowledge of the type of inhibition. By plotting apparent dissociation constants obtained for Hirunorm IV and Hirunorm V as a function of chromogenic substrate concentrations (Fig. 3), a linear dependence was obtained. This

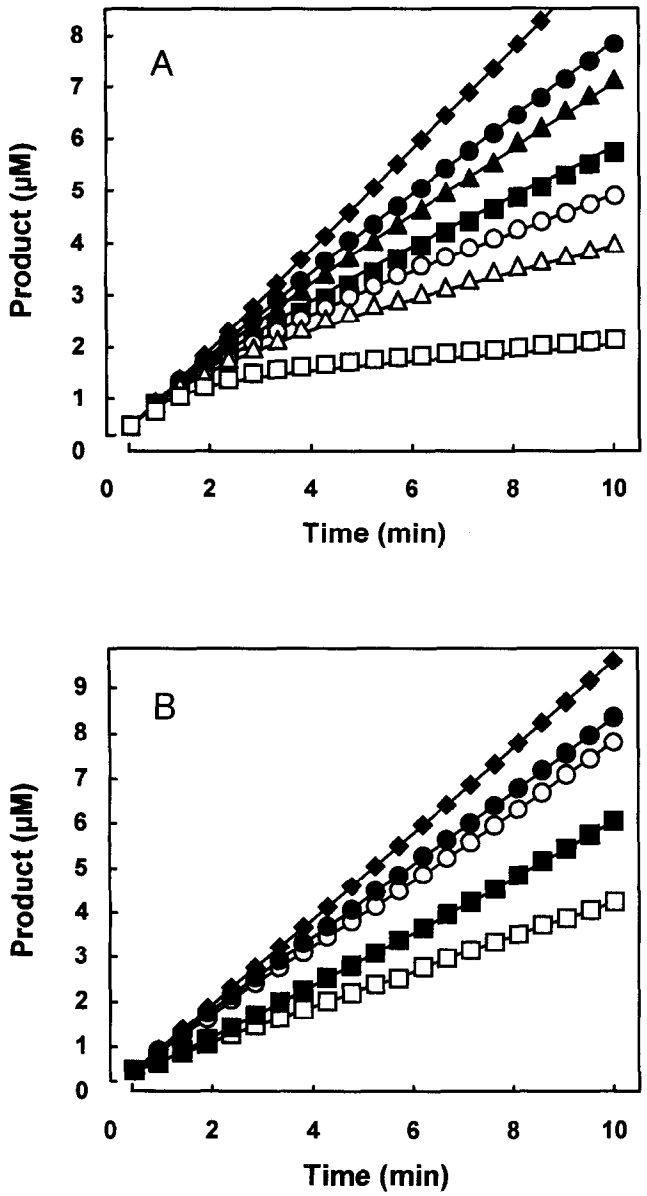

FIG. 1. Time-course of thrombin inhibition by r-hirudin, Hirunorm IV, and Hirunorm V. (A) Slow tight-binding inhibition of human thrombin by r-hirudin. Assays were performed as described in Materials and Methods, in 0.01 M Hepes/0.01 M Tris buffer, pH 7.4, containing 0.4 M NaCl and 0.1% PEG6000 in the presence of 100 μM Chromozym-TH and 0.14 nM thrombin. The concentrations of r-hirudin were 0 (♦), 0.04 (●), 0.08 (▲), 0.12 (■), 0.16 (○), 0.20 (△), 0.40 (□) nM. To evaluate K_I' and K_I, the steady-state velocities obtained from the linear portion of the curves were analyzed as described in the text by using a K_m of 7.5 μM for Chromozym-TH. The curves shown in the figure were drawn by using Eqn (3) and values of 2.4 pM and $3.84 \cdot 10^{-2}$ $pM^{-1} \cdot min^{-1}$ for K_I and k_i, respectively. (B) Fast tight-binding inhibition of thrombin by Hirunorm IV and Hirunorm V. Assays were performed as described in Materials and Methods in 0.01 M Hepes/0.01 M Tris buffer, pH 7.4, containing 0.4 M NaCl and 0.1% PEG6000 with 100 μM Chromozym-TH and 0.14 nM thrombin in the absence (♦) or in the presence of Hirunorm IV (open symbols) or Hirunorm V (closed symbols). The concentrations of inhibitors were 2 nM (circles) and 8 nM (squares).

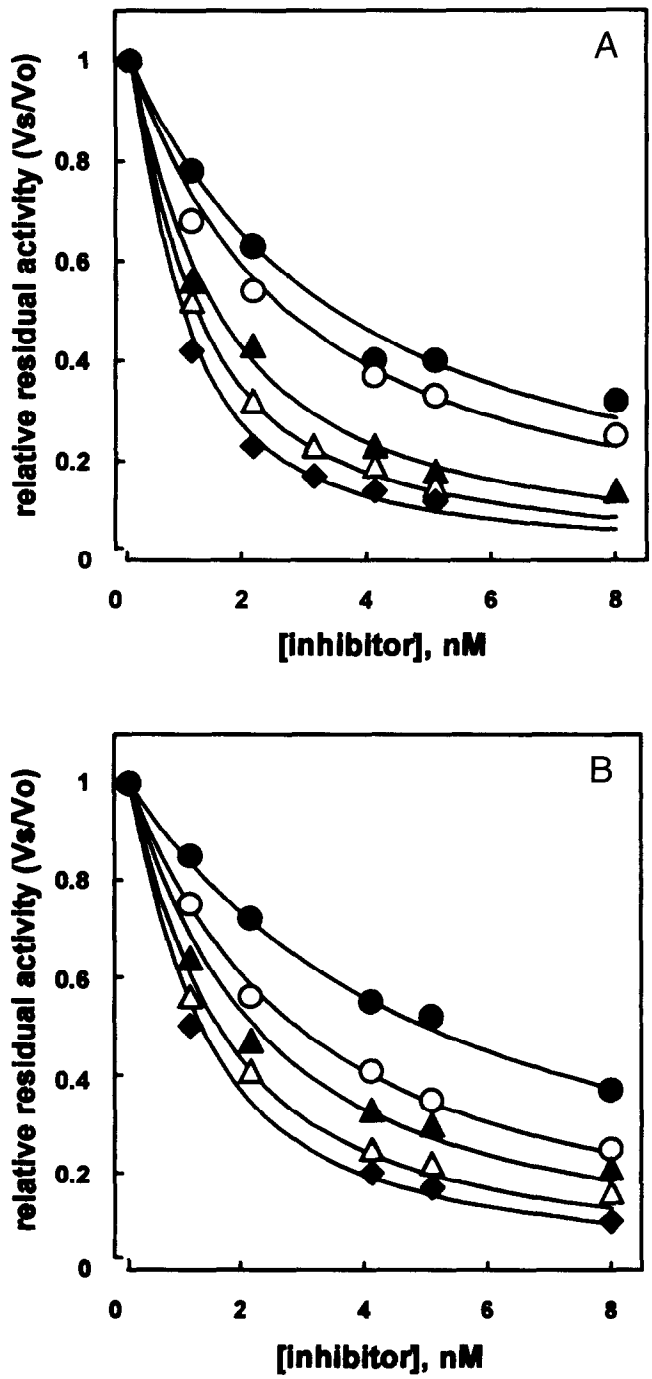

FIG. 2. Effect of Hirunorm IV and Hirunorm V on the steady-state velocity of the thrombin-catalyzed reaction. Assays were performed as described in Materials and Methods, with a concentration of 0.75 nM thrombin at fixed concentrations of Chromozym-TH. The observed steady-state velocities were fitted to Eqn (1) by nonlinear regression analysis to determine K_I' at each substrate concentration. The calculated K_I' (standard errors comprised between 2 and 16%) were used for drawing the lines in the figure. (A) refers to Hirunorm IV (●, 100 μM; ○, 84 μM; ▲, 40 μM; △, 25 μM; ♦, 16 μM Chromozym-TH). (B) refers to Hirunorm V (●, 100 μM; ○, 50 μM; ▲, 32 μM; △, 20 μM; ♦, 13.4 μM Chromozym-TH).

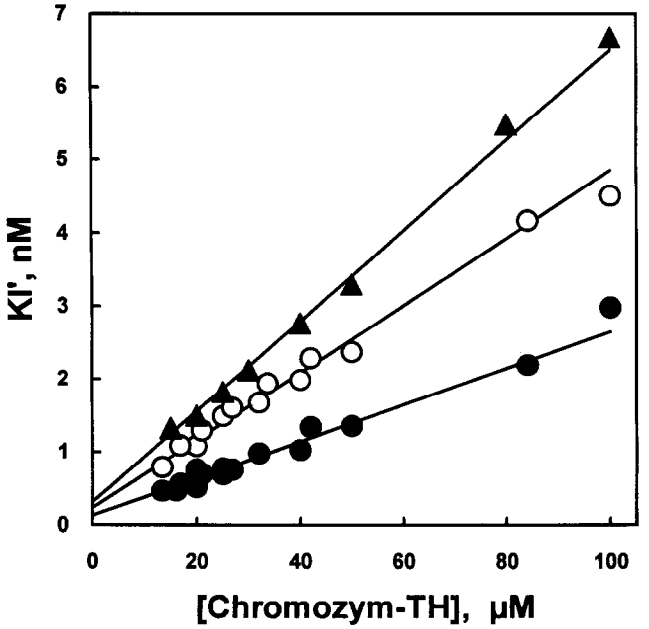

FIG. 3. **Effect of substrate concentration on the apparent dissociation constants for thrombin inhibitors. Values of K_I' for Hirunorm IV (●), Hirunorm V (○), and Hirulog-1 (▲) obtained as described under Fig. 2 (standard errors comprised between 2 and 19%) were plotted vs the concentration of substrate Chromozym-TH used in the enzyme assays. Straight lines are the best-fit curves drawn according to Eqn (2) (see Materials and Methods).**

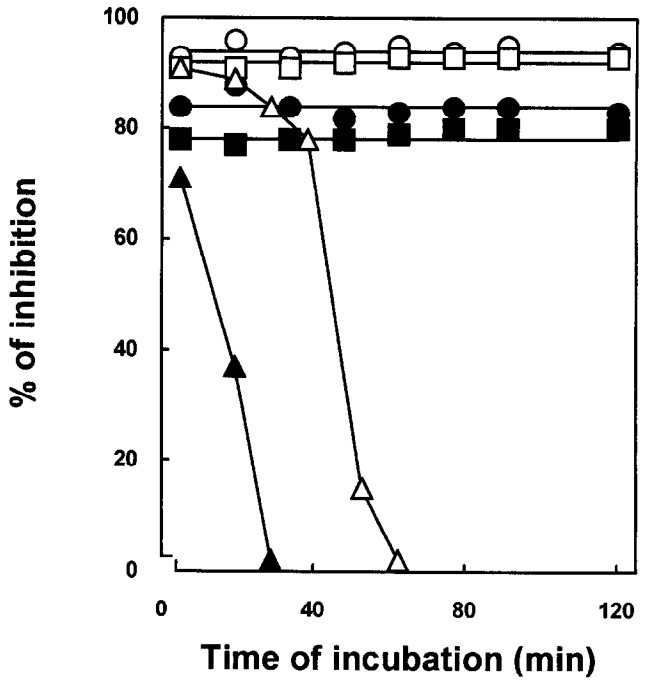

FIG. 4. **Stability of Hirunorm IV, Hirunorm V, and Hirulog-1 as thrombin inhibitors. Human thrombin (1.58 nM) was incubated in the presence of Hirunorm IV (circles), Hirunorm V (squares), and Hirulog-1 (triangles), which were present in the incubation mixture at the concentrations of 10 nM (closed symbols) and 40 nM (open symbols). At indicated times, aliquots of 250 μL were withdrawn from the incubation mixtures and assayed for thrombin amidolytic activity. Data are reported as % inhibition with respect to thrombin activity measured in samples incubated in the absence of inhibitor. Standard deviations evaluated by 3 different experiments for Hirunorm peptides and Hirulog-1 measurements were less than 2% and 15%, respectively.**

finding, which implies that Hirunorm IV and Hirunorm V were competitive inhibitors of thrombin in combination with the K_m value of 5.2 μM determined for the chromogenic substrate Chromozym-TH, made it possible to determine the dissociation constants of the thrombin-inhibitor complex for both Hirunorm IV and Hirunorm V. In Table 1, dissociation constants for Hirunorm IV, Hirunorm V, Hirulog-1 and *r*-hirudin are reported. The value here reported for *r*-hirudin is in good agreement with previously reported values [8]. The dissociation constants obtained for Hirunorm IV and Hirunorm V are among the lowest values reported for synthetic peptide inhibitors of thrombin, being lower than the dissociation constant of Hirulog-1. All the synthetic peptides tested in this study showed three orders of magnitude lower affinity for human thrombin with respect to *r*-hirudin, whose complex with thrombin is essentially irreversible. However, this major difference between *r*-hirudin and Hirulog-1 in the basic *in vitro* test is greatly diminished (to an approximate factor of 10) when the compounds are tested in *in vivo* models, both in animals and in humans [13]. This also holds true for the Hirunorm peptides in animal models [27] and indicates that the apparently lower affinities of the thrombin-inhibitor complexes as compared to *r*-hirudin are unlikely to compromise the therapeutic efficacy of the peptides.

TABLE 1. Kinetic parameters of thrombin inhibition by Hirunorm IV, Hirunorm V, Hirulog-1 and *r*-Hirudin determined by Chromozym-TH amidolytic assay and fibrinogen clotting assay

	Chromozym-TH [K_I (pM)]	Fibrinogen [K_I (pM)]
Hirunorm IV	134 ± 14	196 ± 89
Hirunorm V	245 ± 16	255 ± 70
Hirulog-1	326 ± 13	317 ± 53
r-Hirudin	0.231 ± 0.034	

The potency of Hirunorm IV and Hirunorm V as thrombin inhibitors was confirmed by data obtained in the fibrinogen clotting assay. This assay measures the end point of a complex phenomenon, in which the thrombin-dependent proteolytic cleavage is only part of the overall process. Nevertheless, by assuming that fibrinogen Aα cleavage by thrombin is rate-limiting [19], the dissociation constants at 37°C for the tested inhibitors from fibrinogen clotting assay data are in good agreement with those obtained by amidolytic activity measurements (Table 1).

A major problem with several thrombin inhibitory peptides is that they are cleaved by thrombin [11], thus reducing their inhibitory properties. Both Hirunorm IV and

Hirunorm V incubated in the presence of thrombin proved to be completely stable, remaining active as inhibitors under conditions determining complete inactivation of Hirulog-1 (Fig. 4). Similar results were obtained for Hirunorm V and Hirulog-1 when the peptides were incubated with thrombin at 37°C and catabolism of the inhibitors was evaluated by HPLC [27]. The increased Hirunorm IV and Hirunorm V stability is likely to be linked to the peculiar tertiary structure of these inhibitors, designed to approach the catalytic site on thrombin in an opposite way to that of substrate-like inhibitors (e.g. Hirulog-1). The stability of both Hirunorm IV and Hirunorm V, in combination with their marked inhibitory efficiency and thrombin specificity [27], indicate the potential for both inhibitors to be successfully used for control of the coagulation processes eventually leading to thrombosis.

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