Selective Inhibition of Human α-Thrombin by Cobalt(III) Schiff Base Complexes

Toshihiko Takeuchi,† Arnd Böttcher,‡ Cindy M. Quezada, Melvin I. Simon, Thomas J. Meade,* and Harry B. Gray*

Division of Biology and the Beckman Institute
California Institute of Technology
Pasadena, California 91125

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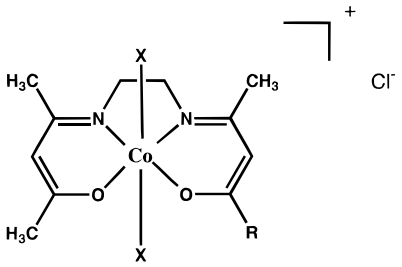
Human α-thrombin, a 34 kD protease associated with the blood coagulation cascade, converts fibrinogen into fibrin, which ultimately forms blood clots.[1] Thrombin activity has been linked to reocclusion of coronary arteries after thrombolytic therapy following heart attacks and therefore is an attractive choice for the development of new inhibitors.[2]

Cobalt(III) Schiff base complexes of class **1** (acacen) (Figure 1)[3] bind histidine residues in active sites and on enzyme surfaces in a random fashion.[4] Spectroscopic and chromatographic evidence indicates that the binding of these complexes is controlled by axial ligand substitution. With several targets, including thrombin, binding of **1** to one or more histidines reduces enzymatic activity.[4c]

To increase inhibitor specificity and potency, a short peptide (-dFPR-) that is known to have a high affinity for the human α-thrombin active site was attached to the chelate (**3**, Figure 1).[5−7] A triglycine linker was inserted between the Schiff base complex and the active-site targeting sequence; the peptide was coupled to the cobalt complex by modification of the Schiff base ligand,[8] followed by activation and coupling to the peptide (NH_2-GGG-d-FPR-CO-NH_2; **5**) via standard solution and solid-phase peptide synthesis techniques.[9]



1. R = CH_3
2. R = CH_2CH_2COOH
3. R = CH_2CH_2CONH-GGG-d-FPR-CO-NH_2
4. R = CH_2CH_2CONH-GGG-d-FPA-CO-NH_2

Figure 1. Structure of cobalt(III) Schiff base complexes: X = NH_3, 2-MeIm; acacen = bis(acetylacetone)ethylenediimine.

The inhibition of thrombin by **3** was evaluated by assaying the activity of the peptide and cobalt complex in both the uncoupled and coupled forms (Figure 2).[10,11] The targeting peptide, **5**, was found to be a reversible inhibitor with $K_i = 290$ μM. Complex **2** does not inhibit thrombin, although it does inhibit other enzymes such as thermolysin.[4c] This lack of inhibition is likely due to unfavorable interactions of the carboxylic acid of **2** with the S1 aspartate located in the arginine binding pocket. In contrast, the **2**−**5** conjugate (**3**) strongly inhibits thrombin:

$$E + CoLX_2 \overset{K_i}{\rightleftharpoons} E{\cdot}CoLX + X \xrightarrow{k_{app}} E{-}CoL + X$$

In the reaction of **3** (1 μM) with the enzyme, a reversible interaction ($K_i = 1.2$ μM) is followed by an irreversible binding step with a pseudo-first-order rate constant (k_{app}) of 3×10^{-5} s^{-1}.

Alanine was substituted for arginine in the peptide-coupled complex (**4**) to test whether the increased inhibition is a result of a *specific* peptide sequence. Alanine is uncharged, and therefore the resulting peptide lacks a critical interaction required for binding to the thrombin active site. Indeed, **4** was found to be a

† Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco 94143-0446.

‡ Present address: BASF AG, Ammoniaklaboratorium, 67056 Ludwigshafen, Germany.

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(6) G = glycine, F = phenylalanine, P = proline, R = arginine, A = alanine, and d refers to the *dextro* isomer of phenylalanine. The peptide was prepared by the Beckman Institute Biopolymer Synthesis Group (Caltech) on *p*-methylbenzhydrylamine (MBHA) resin using *N*-*tert*-butyloxycarbonyl (Boc) amino acid derivatives for Merrifield solid-phase synthesis on an ABI Model 430A peptide synthesizer.

(7) Human α-thrombin contains a Ser195-His57-Asp102 catalytic triad and another aspartate (Asp189) in the substrate-binding region.[5b]

(8) Two equivalents of ethylenediamine were added to a solution of 1 equiv of 4,6-dioxoheptanoic acid in CH_2Cl_2. An insoluble condensation product was isolated and characterized. This material was reacted with 2 equiv of 2,4-pentanedione in absolute ethanol for 6 h and filtered. Successive reactions were performed as previously described and the final product purified by ion exchange or flash chromatography.[4c] The structure of the desired product, **2**, was confirmed by ^1H and ^{13}C NMR, and X-ray crystallographic analysis.

(9) The starting materials (**1**, **2**) and products (**3**−**5**) were characterized by HPLC, MALDI/TOF, and electrospray mass spectrometry.

(10) Human α-thrombin (3037 NIH units/mg) and the substrate Spectrozyme TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide-diacetate salt) were purchased from American Diagnostica. Enzyme-catalyzed hydrolysis rates were measured spectrophotometrically by using a Hewlett-Packard HP8452A diode array spectrophotometer equipped with a HP89090A peltier temperature control accessory. The peptidase activities of thrombin were determined by following the increase in absorption at 406 nm due to the enzymatic hydrolysis of Spectrozyme TH. Initial velocities were determined for ≤10% of the reaction. All assays were performed in 10 mM Tris, 10 mM HEPES, 0.1% poly(ethylene glycol), 0.5 M NaCl, pH 7.8 (run buffer), using 3.07×10^{-9} M thrombin and 40 μM substrate concentration. Irreversible thrombin assays were performed by incubating thrombin *in run buffer to* yield a final enzyme concentration of 1.54×10^{-7} M thrombin and a cobalt concentration (**3**) of 1 μM. These samples were incubated at 25 °C together with controls where the cobalt complex was absent. 20 μL aliquots of these solutions were assayed periodically for residual enzyme activity by dilution of the solution to 3.07×10^{-9} M thrombin, followed by addition of substrate to 40 μM. The percent activities reported are in reference to a thrombin control, which did not lose activity during the course of the experiments.

(11) K_i for the peptide was determined by plotting v_0/v_i versus [I] ($v_0/v_i = [I]/K_i(1 + [S]/K_m) + 1$), using a K_m value of 2.45×10^{-6} M for Spectrozyme TH (from: Sonder, S. A.; Fenton, J. W., II. *Clin. Chem.* **1986**, *32*, 934−937). The determinations were performed with three different concentrations of Spectrozyme (10, 40, and 100 μM) and peptide concentrations ranging from 1 to 20 mM. The IC_{50} value for **3** at time 0 is 1.3 μM. The pseudo-first-order rate constant for inactivation (k_{app}) was calculated from the initial rates of inhibition according to the following: $\ln(E_1/E_2) = k_{app}(T_2 - T_1)$, where E_1 and E_2 are the observed activities at times T_1 and T_2. The K_i of **3** is an upper limit based upon IC_{50} measurements, where $IC_{50} = K_i(1 + \{[S]/K_m\})$ (from: Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharm.* **1973**, *22*, 3099−3108).

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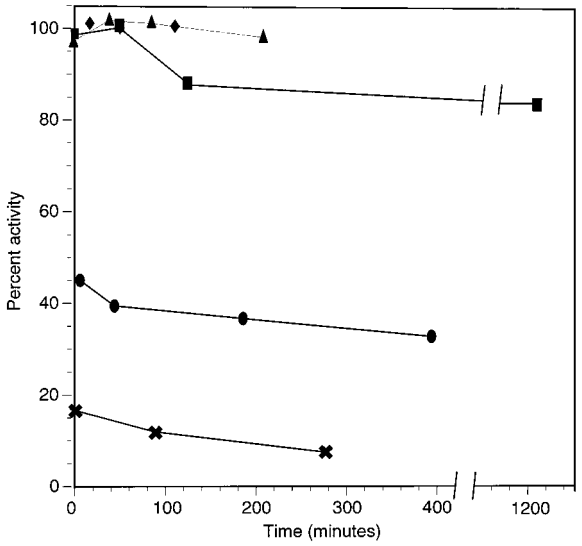

Figure 2. Inhibition assay of human α-thrombin: 1 μM **3** (●), 100 μM **3** (×), $K_i = 1.3$ μM, $k_{app} = 2 \times 10^{-5}$ s^{-1}; 1 μM **1** (■), $K_i > 10^3$ μM; 100 μM **2** (▲), $K_i > 10^3$ μM; 1 μM **5** (◆), $K_i = 2.9 \times 10^2$ μM.

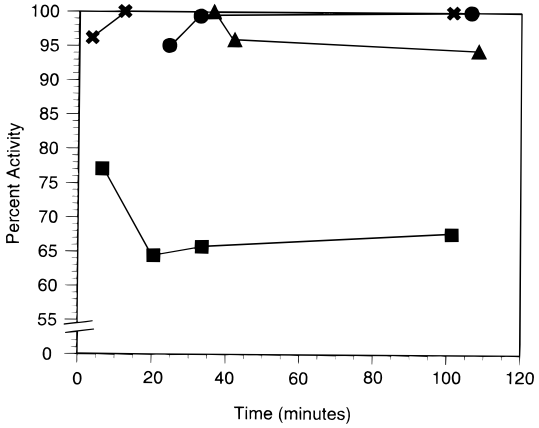

Figure 3. Selective inhibition of thrombin by **3** (■) in the presence of thermolysin (▲) and chymotrypsin (●). Assay of thrombin and 1 μM **4** (×).

very poor inhibitor (Figure 3), consistent with the proposal that there is a specific interaction of the dFPR-coupled cobalt complex (**3**) with the enzyme.

The selectivity of **3** was investigated in a mixture of enzymes containing thrombin, thermolysin, and chymotrypsin (Figure 3). Although **1** can inhibit all three enzymes, **3** should be selective for thrombin, since thermolysin and chymotrypsin only have affinity for peptides with hydrophobic side chains. A cocktail of thrombin, thermolysin, and chymotrypsin was prepared and made 1 μM in **3**.[12] No inhibition of chymotrypsin and thermolysin was observed, while thrombin was inhibited at this concentration. In an experiment in which thrombin was incubated with 100 μM leupeptin, a competitive inhibitor ($K_i = 8.36$ μM)),[13] followed by treatment with **3** (1 μM) and removal of excess inhibitor, enzymatic activity was not diminished. This finding indicates that binding of **3** in the active-site region leads to the observed inhibition of the enzyme.

The active-site target of **3** has not been identified, owing to the acid lability of the cobalt−enzyme complex. The cobalt complex may interact reversibly with the peptide backbone or one or more accessible side chains in the active site. This reversible interaction accounts for the drop in K_i from peptide **5** to the peptide-coupled complex **3**. However, the slow reaction leading to irreversible inhibition may involve substitution at the axial positions of the Co(III) complex; in the fully inhibited enzyme, it is likely that protein ligands occupy these sites.[3,4]

The strategy of linking enzyme-directed groups to cobalt chelates should be applicable to other systems of therapeutic interest. Indeed, complex **1** has shown promise as a clinically useful treatment of ocular infections of herpes simplex virus type 1 (HSV-1).[14] Thus, if the mechanism of HSV-1 inhibition is through interaction with the viral serine protease,[15,16] then second generation viral inhibitors may be developed by linking active-site-directed groups against the viral protease to the cobalt chelate. In the case of thrombin inhibition, modification of **3** could produce inhibitors of clinical interest.

In summary, we have shown that the reaction of an active-site-directed peptide linked to a cobalt chelate leads to selective, irreversible inhibition of thrombin. Further work on such peptide-chelate conjugates could yield powerful and highly selective inhibitors toward a variety of enzyme targets.

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(12) Cocktail assays were performed with 3 nM thrombin assayed with SpectrozymeTH as described above.[11] Thermolysin (Calbiochem) and chymotrypsin (TLCK treated chymotrypsin, Sigma) concentrations in the cocktail were 50 nM and 8 nM, respectively. Thermolysin activity was assayed by following the decrease in absorption at 346 nm due to the enzymatic hydrolysis of 2 mM *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide (FAGLA) (Sigma). Chymotrypsin activity was assayed by following the increase in absorption at 410 nm due to the enzymatic cleavage of 200 nM of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide [SAAPFpNa].

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