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

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Abstract—Cobalt(III) Schiff base complexes have been shown to inhibit the replication of the ocular herpes virus. It is well known that these complexes have a high affinity for nitrogenous donors such as histidine residues, and it is possible that they bind to (and inhibit) an enzyme that is crucial to viral replication. In model studies, we have found that $[Co(acacen)(NH_3)_2]^+$ is an effective irreversible inhibitor of thermolysin at millimolar concentrations; it also inhibits human α-thrombin. Axial ligand exchange with an active-site histidine is the proposed mechanism of inhibition. The activity of thermolysin and thrombin can be *protected* by binding a reversible inhibitor to the active site before addition of the cobalt(III) complex.

Introduction

It has been shown that cobalt(III) Schiff base complexes (Fig. 1) are potent antiviral drugs.[1] While the mechanism of antiviral activity is not yet known, our previous studies have shown that complexes such as $[Co(acacen)(NH_3)_2]^+$ bind to histidine residues of proteins and model peptides.[2,3] ^1H NMR spectra of the isolated products of this reaction reveal that the Co(III) complexes are bound to the nitrogen of the imidazole ring of a histidine residue.[3] Since free histidines are essential for the catalytic activity of many enzymes, a possible mechanism of the observed antiviral activity is through inhibition of a histidine containing viral enzyme that is crucial for replication.

A number of protein families have histidine residues at or near the active site and include serine and cysteine proteases, and RNAses. Enzymes within these families are of therapeutic interest as anticancer, antithrombolytic, antiparasitic, and antiviral targets.[4] Therefore, the development of cobalt(III) Schiff base complexes as antienzymatic therapeutics is of interest due to the wide range of potential targets. This report focuses on the reactions of the cobalt complexes with thermolysin and thrombin and an analysis of the mechanism of inhibition. These two enzymes were chosen because: (1) they contain active site histidines that are crucial to enzyme catalysis, (2) they are of therapeutic interest, and (3) both have been extensively studied. Thermolysin is a 34.6-kDa, zinc-containing neutral metalloendoprotease isolated from *Bacillus thermoproteolyticus*. The amino acid sequence[5] and three dimensional structure[6] are known. The enzyme has received a great deal of attention because it serves as an excellent model for pharmacologically and biologically interesting members of this class of protease.[7] Importantly, it has been shown that chemical modification of the active-site histidine results in enzyme inactivation.[8]

Thrombin is a serine protease that has received attention because of its central role in the blood coagulation cascade.[9] Thrombin converts fibrinogen into fibrin, which crosslinks to form blood clots. In addition, it is involved in the activation of other blood coagulation factors such as V, VIII, XIII, and protein C. Thrombin activity is thought to play a role in the reocclusion of coronary arteries after thrombolytic therapy following heart attacks.[10]

Results and Discussion

Kinetics of inactivation

Concentration-dependent irreversible inhibition was achieved by the addition of $[Co(acacen)(NH_3)_2]Cl$ to thermolysin and thrombin (Fig. 2). In contrast to the relatively rapid and more potent inhibition of thrombin, thermolysin inhibition requires much higher concentrations of the cobalt(III) complex, and the rate of inhibition is characterized by a relatively rapid loss of activity, followed by a slower step. Molecular modeling suggests

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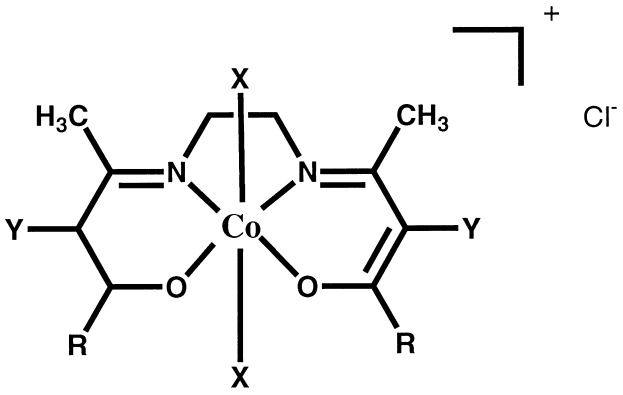

Figure 1. Cobalt(III) Schiff base complexes: X=NH_3, Im, 2-MeIm; R=CH_3, CF_3; Y=H, Cl; acacen = bis(acetylacetone)ethylenediimine.

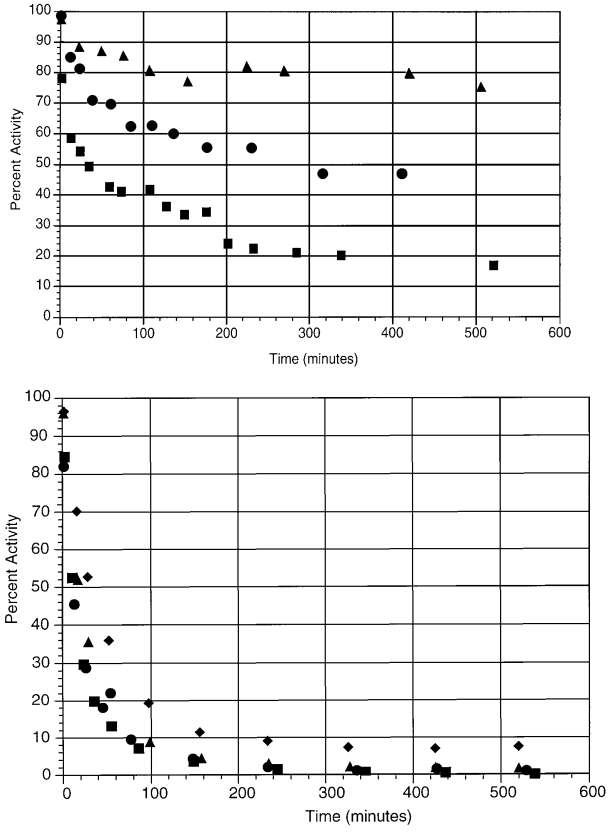

Figure 2. Enzyme inhibition by [Co(acacen)$(NH_3)_2$]Cl: (upper) thermolysin, with [Co(III)] = 5 mM (■), 1 mM (●), and 0.2 mM (▲); (lower) human α-thrombin, with [Co(III)] = 500 μM (■), 200 μM (●), 80 mM (▲), and 25 μM (◆).

that docking of the cobalt complex to the active site of thrombin is more favorable than the interaction with thermolysin, due to unfavorable steric interactions of the Schiff base ligand with the zinc. Thus, for inhibition of thermolysin, higher concentrations of the inhibitor may be necessary to overcome the steric interactions at the active site.

The binding of the cobalt(III) complex to a histidine at the active site results in the loss of zinc from thermolysin. This result is consistent with the interaction of these complexes with zinc finger peptides, where loss of zinc was rapid upon exposure to the Co(III) complexes.[3] Purification of inactivated thermolysin was performed using FPLC gel-filtration chromatography to separate unbound cobalt complex from the Co-modified enzyme. Atomic absorption measurements of the inactivated thermolysin confirmed that zinc had been removed. The rapid initial loss of thermolysin activity may result from binding of the cobalt complex to the active site, followed by loss of zinc in the slow step in the inhibition profile.

Active-site protection

There was no detectable loss of enzyme activity due to irreversible inactivation upon treatment of thermolysin with the strong competitive inhibitor phosphoramidon ($K_i = 32$ nM) in the presence of 5 mM [Co(acacen)$(NH_3)_2$]Cl. Similarly, thrombin activity is protected from cobalt inhibition by leupeptin, a competitive inhibitor ($K_i = 8.36$ μM). The results of these experiments suggest that [Co(acacen)$(NH_3)_2$]Cl inactivates each of the enzymes by binding to the active site.

Characterization of Co(III) thermolysin

Spectroscopic characterization of the inhibited thermolysin revealed two major products: a modified protein with two Co(III) complexes/enzyme and a second product with three Co(III) complexes/enzyme. The completely inactivated product had three cobalt complexes bound to the enzyme. These cobalt complexes remain bound to the enzyme for a period of several weeks, and no recovery of enzyme activity is observed. Since thermolysin is completely active when two cobalt complexes are bound, but completely inactive when three cobalt complexes are bound, these results suggest that binding of one cobalt complex results in complete inactivation of the enzyme. Furthermore, since the binding of the third cobalt complex is prevented by an active-site inhibitor, it is likely that the inhibition of thermolysin is due to the binding of a single inhibitor molecule at the active site. UV–vis spectroscopy shows the characteristic binding of the cobalt acacen complex to histidines; thus, the likely target for the binding of the inhibitor to thermolysin is His231 at the active site. (Although the binding ratios were inconclusive due to incomplete saturation of all binding sites, the observation of leupeptin active-site protection strongly suggests the same mechanism for thrombin inhibition.)

Ligand exchange mechanism of enzyme inhibition

Since the Co(III) complex is bound to a histidine at the active site of the enzyme, a simple ligand exchange mechanism of inhibition is likely. Substitution reactions of $[Co(acacen)L_2]^+$, where L is exchanged with an incoming ligand L′, are expected to be dissociative processes.[11,12] For a dissociative mechanism, the rate-determining step is the loss of ligand, forming a transient five-coordinate complex, followed by rapid binding of the incoming ligand. An associative mechanism is unlikely for these octahedral complexes, owing to the steric

crowding around the Co(III) coordination sphere and lack of suitable orbitals for the bonding of incoming ligands.[12]

If ligand substitution is the rate-limiting step in enzyme inhibition, then factors that increase the rate of axial ligand loss from the cobalt complex should increase the rate of inactivation. Ligand substitution can be accelerated by introducing unfavorable steric interactions between L and acacen or by the use of weaker donors such as ammonia (NH_3). The use of sterically bulky ligands increases the rate of ligand substitution by reducing the activation energy for ligand dissociation. When the axial ligand is imidazole, ligand exchange is unfavorable. Figure 3 shows the irreversible inactivation of thrombin upon incubation with $[Co(acacen)L_2]^+$, where L = imidazole (Im), NH_3, and 2-MeIm (the inhibition profile for L = 2EtIm is virtually identical with that for $L = NH_3$).

When imidazole is the axial ligand, there is little or no loss of enzymatic activity. However, when $L = NH_3$, 2-MeIm or 2EtIm, there is pronounced inhibition of thrombin. These findings support a mechanism in which a weak axial ligand bound to the cobalt complex exchanges with the imidazole of an enzyme histidine residue, resulting in the loss of activity:

$$[Co(acacen)L_2]^+ \longrightarrow [Co(acacen)L]^+$$
$$+L \xrightarrow{E} \left[Co(acacen)(L)^+(E)\right]$$

Effect of electronic tuning of the Schiff base ligand

We have investigated enzyme inhibition after modification of the Schiff base ligand with various electron donating and withdrawing groups. Substitution of the vinylic hydrogen (Fig. 1) with chlorine increases the size of the complex and simultaneously decreases the electron density due to the electron withdrawing nature of the chlorine atom. This change results in a slight decrease in inhibition for $[Co(Cl\text{-}acacen)(NH_3)_2]Cl$ when compared with the unsubstituted complex. Enzymatic activity is not diminished upon incubation of 500 μM $[Co(trifluoroacacen)(NH_3)_2]Cl$ with thrombin and thermolysin. The lack of inhibition is likely due to the slow rate of ligand substitution in the trifluoroacacen species; indeed, UV–vis analysis of a solution of $[Co(trifluoroacacen)(NH_3)_2]Cl$ with imidazole shows that $[Co(trifluoroacacen)(Im)_2]^+$ is not formed. In contrast, incubation of $[Co(acacen)(NH_3)_2]Cl$ with excess imidazole results in a UV–vis spectrum identical with that of $[Co(acacen)(Im)_2]^+$. The observed decrease in the ligand exchange rate in the trifluoroacacen complex is expected, because the transition state involving the loss of NH_3 is destabilized in the more positive cobalt(III) center.

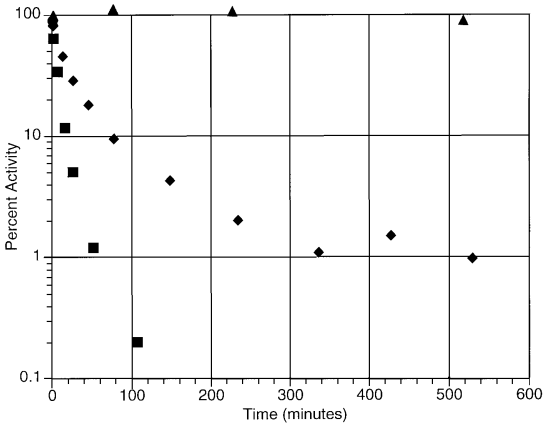

Figure 3. Inhibition of human α-thrombin by 80 μM $[Co(acacen)L_2]Cl$: L=Im (▲), NH_3 (◆), 2-MeIm (■).

In summary, our studies show that cobalt(III) Schiff base complexes can irreversibly inhibit thermolysin and thrombin by binding to the enzyme active site. The data suggest that the mechanism of inhibition is the loss of an axial ligand from the coordinatively saturated $[Co(acacen)L_2]^+$ species, followed by binding to an active-site histidine, resulting in an inactive enzyme-Co(III) complex. Inhibition of thrombin requires much less cobalt complex than the inhibition of thermolysin; this increased potency is most likely due to greater accessibility of the histidine in the thrombin active site. Finally, the reactivity of the cobalt(III) complexes can be electronically tuned by varying the nature of the axial ligand, or by altering the periphery of the Schiff base.[13]

Experimental

Thermolysin (recrystallized 3 times from DMSO) was obtained from Calbiochem and dissolved in 0.1 M Tris (Sigma), 2.5 M NaBr (Aldrich), and 10 mM $CaCl_2$ (Aldrich), pH 7.2. This solution was further purified using gel filtration chromatography on an FPLC instrument using a Superdex 75 column (Pharmacia) equilibrated with 0.1 M Tris, 0.1 M NaBr, 0.01 M $CaCl_2$, pH 7.2 (henceforth described as 'tris run buffer'). This stock solution was stored at 4 °C and the enzyme concentration was determined by $E_{1\%}^{280} = 17.65$[14] and a molecular weight[15] of 34.6 kDa. UV–visible spectroscopy was performed using a Hewlett Packard HP8452A diode array spectrophotometer equipped with a HP89090A Peltier temperature control accessory. N-[3-(2-Furyl)acryloyl]glycyl-L-leucinamide (FAGLA) (Sigma) was used as thermolysin substrate. A stock solution of FAGLA (4.0 mM) was prepared by dissolving the substrate in dimethylformamide (EM Science) (DMF) and diluting it with buffer to a final concentration of 0.1 M Tris, 0.1 M NaBr and 10 mM $CaCl_2$, pH 7.0 (final concentration of DMF, 2.5%). Phosphoramidon (N-(α-L-rhamnopyranosyloxyphospho)-L-leucyl-L-tryptophan) was obtained from Calbiochem. Human α-thrombin (3037 NIH units/mg) and substrate Spectrozyme TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide-diacetate salt) were obtained from American Diagnostica. All other reagents were of the highest quality available from Sigma unless otherwise noted.

Preparation of thrombin stock solutions

Human α-thrombin (1 mL of 30 μM enzyme in 0.75 M sodium chloride solution) was divided into 100 μL aliquots and diluted to 10 mL using aqueous 0.75 M NaCl. These samples were further divided into 1 mL aliquots and stored at −80 °C. Note that samples should not be stored at −20 °C, which is close to the eutectic point for the thrombin–salt mixture, because freeze–thaw cycling may inactivate the enzyme.

Synthesis of N,N′-ethylenebis(acetylacetoneimine) [acacen]

To 500 mL of ethanol were added 500 mL of 2,4-pentanedione (Aldrich). To this solution, 162.5 mL of ethylenediamine were added dropwise using an addition funnel and allowed to react for 1 h at 60 °C. The ligand was crystallized from solution at 4 °C, filtered, and triturated 3x with anhydrous diethyl ether (EM Science), yielding a white solid, MP 110.1–111.1 °C.

Synthesis of N,N′-ethylenebis(trifluoroacetylacetoneimine) [trifluoroacacen]

The compound was prepared as described above with 1,1,1-trifluoro-2,4,-pentanedione in place of 2,4-pentanedione.

Synthesis of [Co(acacen)L_2]Cl (L = 2-Me-Imidazole, Imidazole, NH_3)

Syntheses of [Co(III)(acacen)L_2] complexes have been reported previously.[16] The preparation of [Co(acacen)$(NH_3)_2$]Cl is described here; similar complexes were prepared in an analogous fashion. Cobalt acetate (EM Science) (249.08 g) was dissolved in 1.75 L of methanol (EM Science) and the solution was filtered through Whatman paper number 1. The ligand was suspended in 150 mL methanol. Nitrogen, dried by passage through a silica gel desiccant column, was bubbled over the reagents for 15 min. The cobalt acetate solution was added dropwise (1/2 h) and the orange-brown solution was left to react at room temperature under nitrogen for 2 h. The flask was opened to air, and when L = Imidazole or 2-MeIm, 2 equivalents of the ligand (Aldrich) were added to the solution. When L = NH_3, anhydrous ammonia gas (Matheson) was bubbled into the solution. After filtration on a sintered-glass Buchner funnel, the mixture was concentrated by rotary evaporation and a solution containing one equivalent of sodium chloride dissolved in a minimum amount of water was added. The resulting brown crystalline powder was filtered, washed with methanol, and dried. The structures of the desired products were confirmed by ^1H and ^{13}C NMR, and X-ray crystallographic analysis.

Enzyme assays

All steady-state enzyme assays were performed at 25 °C using the spectrophotometric method of Feder and Schuck.[17] For all assays, the concentrations of enzyme and substrate were 50 nM and 2.0 mM, respectively. The peptidase activities of thermolysin were determined by following the decrease in absorption at 346 nm due to the enzymatic hydrolysis of FAGLA. Initial velocities were determined for < 10% of the reaction.

Treatment of thermolysin with [Co(acacen)L_2]Cl

A stock solution of thermolysin was mixed with the cobalt(III) complex and dissolved in tris-run buffer to yield a final enzyme concentration of 10 mM. The cobalt concentration was varied from 0.2 to 5 mM. The enzyme–cobalt complex solutions were incubated at 25 °C for several hours and, as a control, enzymes were assayed in the absence of the cobalt complexes. Aliquots of these solutions (5 mL) were assayed for residual enzyme activity by their addition to a cuvette containing 495 mL of run buffer and 500 mL of FAGLA stock solution where the absorption at 346 nm was monitored.

Thrombin activity assays

Enzyme-catalyzed hydrolysis rates were measured spectrophotometrically using a Hewlett Packard HP8452A diode array spectrophotometer equipped with a HP89090A peltier temperature control accessory. The peptidase activity of human α-thrombin was determined by monitoring the decrease in absorption at 406 nm due to the enzmatic 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% polyethylene glycol, 0.5 M NaCl, pH 7.8 (run buffer) using 3.07 nM thrombin and substrate concentrations ranging from 1 to 50 mM, depending upon the experiment.

Treatment of thrombin with [Co(acacen)L_2]Cl

A stock solution of human α-thrombin was mixed with the cobalt complex and dissolved in run buffer to yield a final enzyme concentration of 3.10 nM and a cobalt concentration varying from 100 to 1 mM (total volume = 992 mL). These solutions were incubated at 25 °C for several hours along with a control lacking cobalt complex. Several vials were assayed for residual enzyme activity by the addition of the sample to a spectrophotometric cuvette and subsequent addition of 8 mL of 5 mM Spectrozyme TH to initiate the reaction.

To determine the irreversible interaction of the enzyme with the cobalt(III) complexes, a stock solution of thrombin was mixed with the cobalt complex dissolved in run buffer to yield a final enzyme concentration of 154 nM thrombin with the cobalt concentration varying from 50 to 500 mM. These samples were incubated at 25 °C for several hours along with a control lacking cobalt complex. Periodically, 20-mL aliquots of these solutions were assayed for residual enzyme activity by their addition to a spectrophotometric cuvette containing 972 mL of run buffer and 8 mL of 5 mM Spectrozyme TH solution.

Active-site protection of thermolysin

Thermolysin (10 M, tris run buffer, pH 7.2) was incubated at 25 °C with [Co(acacen)$(NH_3)_2$]Cl (5 mM) in the

presence and absence of the inhibitor phosphoramidon (50 mM), which has a reported K_i of 32 nM at pH 7.[18] Phosphoramidon binds to thermolysin at the active site, and this enzyme-inhibitor complex has been crystallographically characterized.[19] After incubation with the cobalt complex overnight, the enzyme was separated from any phosphoramidon and cobalt complex that was not covalently attached to the enzyme using gel filtration chromatography on a FPLC instrument using a Superdex 75 column (Pharmacia) equilibrated with 0.1 M Tris, 5 mM $CaCl_2$, pH 9. The resulting solution was transferred into tris run buffer using a PD-10 column (Pharmacia), and characterized spectrophotometrically.

Active-site protection of thrombin

Thrombin (300 nM in thrombin run buffer) was incubated at 25 °C with 5 mM [Co(acacen)$(NH_3)_2$]Cl in the presence and absence of 100 μM leupeptin, a competitive inhibitor of thrombin ($K_i = 8.36$ μM).[20] After overnight incubation, the enzyme was separated from excess inhibitor using a Superdex 75 column equilibrated with thrombin run buffer that was supplemented with 1 M guanidine hydrochloride. The enzyme was transferred into thrombin run buffer using a PD-10 column and assayed for activity.

Analysis of [Co(acacen)L_2]Cl:enzyme binding ratios

The cobalt complex:enzyme binding ratios were determined using UV–visible spectroscopy. The concentration of the cobalt complex in the solution was evaluated by measuring the absorbance at 338 nm, using an extinction coefficient of 6670 $M^{-1}\,cm^{-1}$, which is the value for [Co(acacen)$(Im)_2$]Cl. The protein absorbance at 280 nm was determined by subtraction of the cobalt complex absorbance at that wavelength. The protein concentration was calculated using $E_{1\%}^{280} = 17.65$ and a molecular weight of 34.6 kDa. This method was found to be accurate to ± 5%.

Atomic absorption measurements

Water was glass-distilled and passed through a primary deionization unit and a 0.2 mm postfilter cartridge (Barnstead). The solution was passed through a mixed-bed resin (Sigma). Plastic containers used for pipette tips and Eppendorf tubes were acid-cleaned by rinsing with 1:40 nitric acid/water (vol/vol) and then by rinsing three times with zinc-free water. Samples were transferred into zinc-free water using a PD-10 column (Pharmacia) that had been washed with 50 mL of 50 mM Tris, 2.5 mM 1,10-phenanthroline, pH 7.2, and then equilibrated with 50 mL of zinc-free water. The resulting solutions were diluted 1:1 with Ultrex II concentrated nitric acid (J. T. Baker), and heated at 60 °C for 6 h. Atomic absorption measurements were made on a Varian (AA-875) graphite furnace atomic absorption spectrophotometer.

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