The interaction of thrombin with several potent and selective α-ketoamide transition state analogs was characterized. L-370,518 (H-N-Me-D-Phe-Pro-4-aminocyclohexylglycyl-N-methylcarboxamide) a potent ($K_i = 90$ pm) and selective (>10-fold versus trypsin) ketoamide thrombin inhibitor was shown to bind thrombin via a two-step reaction wherein the initially formed thrombin-inhibitor complex ($E\text{I}_1$) rearranges to a more stable, final complex ($E\text{I}_2$). A novel sequential stopped-flow analysis showed that $k_{-1}$, the rate constant for dissociation of $E\text{I}_1$, was comparable to $k_2$, the rate constant for conversion of $E\text{I}_1$ to $E\text{I}_2$ (0.049 and 0.035 s$^{-1}$, respectively) indicating that formation of the initial complex $E\text{I}_1$ is partially rate controlling. Replacement of the N-terminal methylenamino group in L-370,518 with a hydroxyn (L-372,051) resulted in a 44-fold loss in potency ($K_i = 4$ nm) largely due to an increase in $k_{-1}$. Consequently in the reaction of L-372,051 with thrombin formation of $E\text{I}_1$ was not rate controlling. Replacement of the P1’ N-methylcarboxamide group of L-370,518 with an azetidylcarboxamido (L-372,228) produced a 58-fold increase in the value of the equilibrium constant ($K_{d}$) for dissociation of $E\text{I}_1$. Nevertheless, L-372,228 was a 2-fold more potent thrombin inhibitor ($K_i = 40$ pm) than L-370,518 due to its 16-fold higher $k_{-2}$ and 10-fold lower $k_{-2}$ values. The desketooamide analogs of L-370,518 and L-372,051, namely L-371,912 and L-372,011, inhibited thrombin via a one-step process. The $K_i$ value for L-371,912 and the $K_{d}$ value for its α-ketoamide analog, L-370,518, were similar (5 and 14 nm, respectively). Likewise, the $K_i$ value for L-372,011 and the $K_{d}$ value for its α-ketoamide analog, L-372,051, were similar (330 and 285 nm, respectively). These observations are consistent with the view that the α-ketoamides L-370,518 and L-372,051 form initial complexes with thrombin that are similar to the complexes formed by their desketooamide analogs, and in a second step the α-ketoamides react with the active site serine residue of thrombin to form a more stable hemiketal adduct.

Our pursuit of novel antithrombotic agents has focused on direct inhibitors of thrombin, a trypsin-like serine proteinase that plays a key role in thrombosis. One strategy for the design of potent thrombin inhibitors is to replace the substrate P1 carbamido group with an electrophilic keto or aldehyde group (1). Cyclotheonamide A is a naturally occurring proteinase inhibitor from a marine sponge (Theonella) that contains such a potency-enhancing keto group (2–4). Cyclotheonamide A is a potent ($K_i = 1$ nm) reversible inhibitor of thrombin (5, 6) and several other trypsin-like proteinases (5, 7). Various α-ketoamide derivatives of tripeptide substrates of thrombin have been prepared in an attempt to identify selective, potent, reversible inhibitors of thrombin (8, 9). Placement of a t-4-AChxGly$^2$ residue at the P1 position in α-ketoamide thrombin inhibitors provided excellent selectivity for thrombin, relative to trypsin (10–12), probably because the S1 specificity pocket of thrombin is larger than that of trypsin (13, 14).

In the present study, we show that α-ketoamide inhibitors containing t-4-AChxGly inactivate thrombin via a two-step reaction, wherein an initially formed weak complex (E$\text{I}_1$) rearranges to a more stable thrombin-inhibitor complex (E$\text{I}_2$) (Scheme I). Inhibitors that inactivate enzymes via a two-step pathway are usually assumed to form E$\text{I}_1$ rapidly in a pre-equilibrium reaction where $k_{-1}$ is assumed to be much greater than $k_{-2}$ (15). Sculley et al. (16) recently discussed the difficulty in distinguishing cases where $k_{-1} \gg k_{-2}$ from those where $k_{-1} \sim k_{-2}$. We now describe a novel sequential stopped-flow analysis that circumvents this difficulty and allowed us to rigorously evaluate kinetic pathways for inactivation of thrombin by a family of active site-directed thrombin inhibitors.

**EXPERIMENTAL PROCEDURES**

Methods for the synthesis of the α-ketoamide and desketooamide derivatives of H-N-Me-n-Phe-Pro-t-4-AChxGly have been reported elsewhere (Table I and Refs. 9–12). The inhibitor concentrations were determined from titration with a known amount of thrombin as described previously (5). Dansylarginine N’-(3-ethyl-1,5-pentanediyiylamido) (DAPA) was obtained from American Diagnostica. Concentrations of DAPA were determined from measurements of absorbance at 330 nm using an extinction coefficient of 4.01 cm$^{-1}$ mm$^{-1}$ (17). The sources of other materials were described previously (5, 8). Proteinase assays were performed at room temperature in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% polyethylene glycol 8000 unless otherwise indicated.

**Determination of Inhibition Constants ($K_i$)**—The inhibition constants were determined as described previously (5, 8, 9). When total inhibitor ([I]$\_i$) and enzyme ([E]$\_i$) concentrations were comparable, the quadratic equation (18–20) for tight-binding inhibitors (Equation 1) was used to calculate the apparent inhibition constant ($K_{d,i}$) from the dependence of substrate hydrolysis on [I]$\_i$ and [E]$\_i$, where $V_i$ and $V_0$ represent the initial rates of substrate hydrolysis in the presence and absence of

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**Scheme I.** One- and two-step pathways for inhibition of thrombin.

| inhibitor, respectively. |
|---------------------------|
| $V/V_o = \frac{[E][I]}{[E][I] + [I] + \frac{[E] + K_i + [E][I]^2 + 4K_i[I]}{2}[E]}$ (Eq. 1) |
| When $[I] \gg [E]$, the variation of $V/I$ with $[I]$ is described by Equation 2. |
| $V/V_o = \frac{1 + [I]/K_I}{1 + [I]/K_I}$ (Eq. 2) |

In assays where the substrate fully equilibrates with inhibitor and enzyme (i.e. analysis of progress curves, see below), Equation 3 relates the apparent inhibition constant, $K_I$, to the intrinsic, final inhibition constant, $K_i$.

$K_i = K_I + [S]/K_o$ (Eq. 3)

In assays where the inhibitor and enzyme were preequilibrated (i.e. before addition of substrate), and the rate of dissociation of inhibitor from enzyme was slow, and didn't occur during the time of the activity assay, the use of Equation 3 is inappropriate and the apparent inhibition constant, $K_I$, is equal to $K_i$. Activity assays for the determination of $K_i$ were routinely performed at $[S] \ll K_i$, hence, $K_i = K_I$ and the decision of when it was appropriate to use Equation 3 were circumvented. The overall dissociation constant ($K_o$) is defined by Equations 4 and 5 for the one- and two-step pathways, respectively (Scheme I).

$K_o = \frac{[E][I]}{[E][I] + [I] + K_i}$ (Eq. 4)

**Determination of Rate Constants for Schemes I and II**—The rate of inhibitor binding to thrombin was followed by displacement of the fluorescent probe $p$-aminobenzamidine from the active site of thrombin. The decrease in fluorescence ($F$) was monitored using an Applied Photophysics stopped-flow spectrometer (DX.17MV) interfaced with an Archimedes 420l computer as described previously (5, 8). Rate constants were derived from analysis of the average of 4–7 replicate traces with 1000–4000 data points per trace. Typically, $p$-aminobenzamidine (100–600 $\mu$m) was mixed with 0.25–1 $\mu$m thrombin prior to reaction with an equal volume of inhibitor (2.5–80 $\mu$m). When decay of the fluorescent signal was monophasic, Equation 6 ("single exponential with floating end point," Applied Photophysics software) was used to evaluate the kinetic parameters $k_{obs1}$.

$F = (F_o - F_{\infty})e^{-k_{obs1}t} + F_{\infty}$ (Eq. 6)

In Equation 6, $F$ is the measured fluorescence at time $t$, $k_{obs1}$ is the apparent first-order rate constant for the approach of $F$ to its final value $F_o$, and $F_{\infty}$ corresponds to the fluorescence at $t = 0$. The dependence of the pseudo-first-order rate constant ($k_{obs1}$) for displacement of $p$-aminobenzamidine is a linear function of $[I]$. Equation 7 was used to obtain $k_{obs1}$, where $[I]_{eff} = [I]/[1 + [S]/K_o]$ and $K_i$, (the equilibrium constant for dissociation of the enzyme-$p$-aminobenzamidine complex) is equal to 47 $\mu$m (5).

$V/O = k_{obs1} - k_{obs2}$ (Eq. 7)

When the decay of fluorescence accompanying probe displacement was a biphasic process, Equation 8 ("double exponential with floating end point," Applied Photophysics software) was used to yield the kinetic parameters $k_{obs1}$ and $k_{obs2}$.

$F = A_1e^{-k_{obs1}t} + B_1e^{-k_{obs2}t} + F_{\infty}$ (Eq. 8)

In Equation 8 $k_{obs1}$ and $k_{obs2}$ are the apparent first-order rate constants for the approach of $F$ to its final value, and $A_1$ and $B_1$ are amplitude terms associated with their corresponding first-order processes. The dependence on $[I]$ of the pseudo-first-order rate constants, $k_{obs1}$ and $k_{obs2}$, for displacement of $p$-aminobenzamidine was a linear and hyperbolic function of $[I]_{eff}$ respectively, and was fit by Equations 9 and 10 for evaluation of $k_1$, $k_2$, and $K_i$.

$k_{obs1} = k_1 + k_2[I]_{eff}$ (Eq. 9)

$k_{obs2} = k_2 + k_2[I]_{eff}/[K_i + [I]_{eff}]$ (Eq. 10)

Stopped-flow experiments also yielded progress curves for thrombin-mediated hydrolysis of Z-GPR-afc (400-nm excitation with a 455-nm emission block) in the presence of inhibitor (5, 8). Briefly, inhibitor was mixed with Z-GPR-afc (22 $\mu$m) prior to reaction with an equal volume of 5–20 $\mu$m thrombin. Substrate depletion was less than 10% during the run. Rates were measured under pseudo-first-order conditions (i.e. [inhibitor] $>>$ [thrombin], [Z-GPR-afc] $>>$ [thrombin], and [Z-GPR-afc] $< K_i$). The best fit for a single exponential decay was "single exponential with steady state" (from the kinetic software package supplied by Applied Photophysics), or alternatively, the data were transferred to Kaleidagraph (version 3.0.5 Abelbeck Software) and fitted using Equation 11. Kaleidagraph utilizes the Levenberg-Marquardt algorithm

$F = V_{st}t + (V_{init} - V_o)(1 - exp(-k_{obs1}t))/k_{obs} + F_o$ (Eq. 11)

for nonlinear least-squares regression. Equation 11 as developed by Williams and Morrison (18) and by Cha (20) is commonly used in the analysis of monophasic progress curves. In Equation 11, $F$ is the measured fluorescence defined as a function of the initial ($V_{init}$ and $V_s$) steady state velocities (change in fluorescence per unit time due to thrombin-catalyzed substrate hydrolysis) and the apparent first-order rate constant ($k_{obs}$) for the approach of enzymic activity to its final value. To monitor directly the time-dependent inhibition of thrombin in the presence of a substrate, the following analysis was employed. At long times ($k_{obs}t \gg 1$) where Equation 11 reduces to Equation 12, $F$ is a linear function of time. Extrapolation of the linear time dependence

$F = V_{st}t + (V_{init} - V_o)/k_{obs} + F_o$ (Eq. 12)

of $F$ at long times to zero time and determination of the difference ($\Delta$) between the values of $F$ on the extrapolated linear plot (Equation 12) and those on the plot describing the time dependence of the experimentally determined values of $F$ (Equation 11) should provide an indication of the time-dependent approach of enzymic activity to its final value as indicated by Equation 13 where $\Delta_s$ is the value of $\Delta$ at $t = 0$.

$\Delta_s = exp(-k_{obs}t)$ (Eq. 13)

When the time-dependent decrease in the parameter $\Delta_s$ was biphasic, the dependence of $F$ on time was fit by Equation 14 where $C$ and $A$ are constants.

$F = V_{st} + C[1 - A exp(-k_{obs}t) - (1 - A)exp(-k_{obs}t)] + F_o$ (Eq. 14)

As in the case of the monophasic process described above, the dependence of $F$ on time should become linear at long times (Equation 15). Extrapolation of the linear portion

$F = V_{st} + C + F_o$ (Eq. 15)

of the $F$ versus time plot to zero time and determination of the differences ($\Delta$) between the values of $F$ on the extrapolated plot and the plot defined by the observed time dependence of $F$ yields a biphasic time dependence parameter $\Delta_s$, as indicated by Equation 16.

$\Delta_s = A exp(-k_{obs}t) + (1 - A) exp(-k_{obs}t)$ (Eq. 16)

It is important to note that the relative magnitude of the amplitude factors $A$ and $1 - A$ reflect the relative amount of hydrolysis products formed in the fast and slow phases and not the relative amount of enzyme inactivated in the slow and fast phases.

When the time-dependent approach of enzymic activity to its final value was monophasic, the dependence of $k_{obs}$ on $[I]_{eff}$ (where $[I]_{eff} = [I]/[1 + [S]/K_o]$) was fit by Equation 7 to obtain $k_1$. When the approach of enzymic activity to its final value was biphasic, the time dependence of fluorescence was fit by Equations 14 or 16 to determine $k_{obs1}$, $k_{obs2}$, and $A$ (the fraction of the biphasic reaction described by $k_{obs1}$). The dependence of $k_{obs1}$ and $k_{obs2}$ on $[I]_{eff}$ was fit by Equations 9–10 to obtain the parameters $k_1$, $k_2$, and $K_i$. The values for $k_1$ and $k_2$ could not always be determined accurately from the fit of the data by Equations 7, 9, and 10. In such cases, other methods were used to determine their values (see below).
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**Scheme II.** Regeneration of active enzyme via the two-step reaction pathway.

The value of $k_2$ was determined from the time-dependent regeneration of free enzyme as measured by the hydrolysis of a fluorogenic substrate (Scheme II). Thrombin was preincubated with inhibitor at a concentration much greater than $K_i$ and for sufficient amount of time to ensure complete formation of $E_2$. After preincubation the enzyme-inhibitor complex was diluted into a solution containing 60 μM t-Phe-Pro-Arg-fluorogenic substrate (IS) > $K_i$, $K_m = 0.3$ μM. The resulting progress curve was fit to Equation 11 to obtain $k_{obs}$, which is equivalent to $k_{cat}$. Equation 17 was used to relate $k_{cat}$ to the rate constants for the two-step pathway depicted in Schemes I and II.

$$k_{cat} = \frac{k_2}{k_1 + k_2}$$ (Eq. 17)

For very potent inhibitors, $k_2$ was exceedingly small and the long times required for complete regeneration of enzyme compromised enzyme stability. In these cases $V_i$ was determined in a separate experiment from an identical dilution of enzyme with substrate. The experimentally determined value of $V_i$ was fixed in Equation 11, the initial data from the progress curve (~3–5 half-lives) was fitted by nonlinear regression to Equation 11 to determine $k_{cat}$.

**Sequential Stopped-flow—**To determine the value of $k_{cat}$, a sequential stopped-flow method was used. Equal volumes of thrombin and inhibitor (II) >> $K_i$ were aged long enough (first mix) to ensure complete formation of $E_1$, but with minimal formation of $E_2$ (see Scheme I). After preincubation (first mix), $E_1$ was diluted (second mix) with an equal volume of DAPA (Scheme III). There is an increase in fluorescence when DAPA binds to the active site of thrombin (excitation 280 nm, emission block 420 nm (17, 21)). A high concentration of DAPA (>40 μM) ensured that the pseudo-first-order rate constant ($k_{cat}$) for the reaction of $E$ with DAPA was much greater than $k_{cat}$ and $k_{on}$; hence, the reaction of thrombin with DAPA is not rate-limiting. In Scheme III the pseudo-first-order rate constant, $k_{obs}$, for regeneration of $E$ from $E_1$, is described by Equation 18.

$$k_{obs} = k_2 - k_1$$ (Eq. 18)

The value of $k_{obs}$ was estimated using Equation 11 to fit the time-dependent increase of fluorescence associated with DAPA binding to the active site of thrombin concomitant with inhibitor displacement. Equa-

**Results**

**Inhibition of Thrombin Catalysis—**Treatment of thrombin with L-371,912 or the corresponding α-ketoamide analog L-370,518 inhibited thrombin-catalyzed hydrolysis of a fluorogenic substrate (Fig. 1). The equilibrium constants ($K_i$) for dissociation of the complexes between thrombin and the des-ketoamide L-371,912 and the ketoamide L-370,518 were 5 ± 0.5 nM and 90 ± 10 μM, respectively. The titrations shown in Fig. 3 and 4A (Table II) indicates that these second-order rate constants are truly equivalent to $k_1$.

The apparent discrepancy between the biphasic inhibition of thrombin-catalyzed substrate hydrolysis and the monophasic p-aminobenzamidine displacement observed in the case of L-370,518 stems from the differences in the two experimental methods. With the elevated L-370,518 concentrations used for the p-aminobenzamidine displacement studies (2.5–25 μM), only a small amount of free enzyme is present at the end of the first phase ($K_i$ is 1.25 μM, see Fig. 4B); hence, the binding of $E$ to $I_1$ is the dominant reaction. The amplitude of the second phase, which is governed by the conversion of residual $E$ to $E_1$ and $E_2$, is too small to measure by the p-aminobenzamidine displacement method. On the other hand, the activity assay can detect the small amount of active enzyme present at the end of the first phase and measure its subsequent decay...
FIG. 1. Determination of the equilibrium constants \( (K_i) \) for dissociation of the thrombin-L-371,912 and thrombin-L-370,518 complexes. Reactions were initiated by addition of 1.25 \( \mu \)M Z-GPR-afc (final concentration) to equilibrated solutions of 0.1 nM thrombin containing
of the value of $k_0$ sufficiently to ensure formation of thrombin which was subsequently mixed with L-370,518. The approach of fluorescence to the requisite condition of rapid equilibrium between $k_0$.

The mean rate constant, 0.084 s$^{-1}$, was obtained from the amplitude of the initial fluorescence change produced upon quenching with DAPA and the use of the following relationship (Equation 19).

$$f_{EU} = (F_i - F_p)/(F_A - F_p)$$

where $F_i$ is the initial fluorescence after aging time $t$, $F_A$ is the initial fluorescence with no aging with L-370,518 (trace A, Fig. 6A), and $F_p$ is the fluorescence after complete aging with L-370,518 (trace F, Fig. 6A). The pseudo-first-order rate constant derived from the slope in Fig. 7A, 6.7 s$^{-1}$, was used to calculate a second-order rate constant, 3.4 $\mu M^{-1}$ s$^{-1}$, from the equation $k_1 = 6.7$ s$^{-1}$/[L-370,518] using the experimental concentration of L-370,518 (2 $\mu M$). As expected, this $k_1$ value is equivalent to that obtained from studies of substrate hydrolysis and probe displacement.

Even at the shortest preincubation times, thrombin was not fully recoverable from $E_1$ as shown by the failure of $trace B$ (aging time 48 ms) in Fig. 6A to approach the final fluorescence value of $trace A$. This observation is consistent with a two-step pathway (Scheme I) where $k_0$ is comparable to $k_1$. At longer preincubation times (Fig. 6A, traces D and E) all of $E$ has reacted with L-370,518 to form $E_1$, or $E_2$; hence, the fraction of enzyme recoverable from $E_1$ is a function of both the ratio $k_2/(k_1+k_2)$ and the time of preincubation. The fraction of enzyme recoverable from $E_1$ ($f_{EU}$) at the end of various aging times could be obtained from the amplitude of the final fluorescence (corrected for time-dependent bleaching) produced upon quenching with DAPA and the use of the following relationship (Equation 20).

$$f_{EU} = (F_i - F_p)/(F_A - F_p)$$

where $F_p$ is the final fluorescence after aging time $t$ and $F_A$ and $F_p$ are described above. Fig. 7B depicts a log plot of the $f_{EU}$ versus aging time and yields a pseudo-first-order rate constant of 0.046 s$^{-1}$ with an intercept at $-0.5$. A plausible interpreta-

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**Fig. 2. Monophasic and biphasic inactivation of thrombin by L-371,912 and L-370,518 under stopped-flow conditions.** Thrombin (20 nM) was mixed with an equal volume of 25 $\mu M$ Z-GPR-afc and 1 $\mu M$ L-371,912 (open squares) or L-370,518 (open circles). The solid lines represent the best fit to the data by Equation 13 (L-371,912) or Equation 16 (L-370,518). Using the values of the rate constants ($k_1$, $k_{-1}$, $k_2$, $k_{-2}$) listed in Table II, a theoretical time dependence of $\Delta F$ (indistinguishable from that defined by the solid lines in the figure) was obtained for the biphasic reaction by Runge-Kutta digital integration of the differential equations describing the two-step reaction pathway in Scheme I. Only 5% of the collected data points are shown for reasons of clarity.

**Use of Sequential Stopped-flow Analysis to Characterize Individual Steps in Inhibitory Pathways**—Fig. 6A depicts the results of a sequential stopped-flow experiment designed to determine the individual rate constants of a two-step pathway. Accordingly, thrombin was mixed with L-370,518 and aged sufficiently to ensure formation of $E_1$, and partial conversion of $E_1$ to $E_2$. The aged mixture was subsequently mixed with DAPA, a fluorogenic ligand that binds tightly to the active site of complexed thrombin. Fig. 6A shows the increase of fluorescence as $E$ is regenerated from $E_1$ and combines with DAPA. When the first reaction mixture is aged long enough for complete conversion of $E_1$ to $E_2$, no reaction with DAPA is observed within the 100-s observation time (Fig. 6A, trace F). Trace A shows the fluorescence/time profile for DAPA-treated thrombin which was subsequently mixed with L-370,518. The absence of a time-dependent change of fluorescence indicated that the rapidly formed thrombin-DAPA complex is not displaceable by L-370,518 over the time and concentration range used in the experiment. The approach of fluorescence to the final value in traces B–E (which represents, in rank alphabetical order, increasing incubation times of L-370,518 and thrombin) was a pseudo-first-order process that when fit by Equation 11 yielded rate constants of 0.086, 0.085, 0.086, and 0.075 s$^{-1}$, respectively. The mean rate constant, 0.084 s$^{-1}$, should be equivalent to $k_{-1} + k_2$ (Equation 18), provided that the pseudo-first-order rate constant for reaction of thrombin with DAPA is greater than $k_{-1} + k_2$ and that trapping of free thrombin with DAPA is operationally irreversible. These conditions appear to be satisfied since (i) the rate constants for displacement of L-370,518 from thrombin by DAPA is independent of the DAPA concentration (data not shown); (ii) the pseudo-first-order rate constant for reaction of DAPA with thrombin is $>1000$ s$^{-1}$ which exceeds $k_1$ and $k_{-1} + k_2$ when DAPA concentrations are $\geq 40 \mu M$ (data not shown). Since $k_{-1} + k_2 = 0.084$ s$^{-1}$, a value 0.049 s$^{-1}$ is obtained for $k_{-1}$ using the previously determined $k_2$ value of 0.035 s$^{-1}$.

At short aging times of thrombin with L-370,518, a burst of fluorescence was evident after the addition of DAPA (Fig. 6A, traces B and C) which is attributed to the fact that conversion of $E + 1$ to $E_1$ was incomplete at short aging times and the uncomplexed $E$ reacted with DAPA during the dead time of the instrument. A log plot of the fraction of uncomplexed $E$ ($f_{EU}$) versus aging time with L-370,518 (Fig. 7A) should give a reliable estimate of $k_1$. The value of $f_{EU}$ at the end of various aging times was obtained from the amplitude of the initial fluorescence change produced upon quenching with DAPA and the use of the following relationship (Equation 19).

$$f_{EU} = (F_i - F_p)/(F_A - F_p)$$

where $F_p$ is the initial fluorescence after aging time $t$, $F_A$ is the initial fluorescence with no aging with L-370,518 (trace A, Fig. 6A), and $F_p$ is the fluorescence after complete aging with L-370,518 (trace F, Fig. 6A). The pseudo-first-order rate constant derived from the slope in Fig. 7A, 6.7 s$^{-1}$, was used to calculate a second-order rate constant, 3.4 $\mu M^{-1}$ s$^{-1}$, from the equation $k_1 = 6.7$ s$^{-1}$/[L-370,518] using the experimental concentration of L-370,518 (2 $\mu M$). As expected, this $k_1$ value is equivalent to that obtained from studies of substrate hydrolysis and probe displacement.

Even at the shortest preincubation times, thrombin was not fully recoverable from $E_1$ as shown by the failure of $trace B$ (aging time 48 ms) in Fig. 6A to approach the final fluorescence value of $trace A$. This observation is consistent with a two-step pathway (Scheme I) where $k_0$ is comparable to $k_1$. At longer preincubation times (Fig. 6A, traces D and E) all of $E$ has reacted with L-370,518 to form $E_1$, or $E_2$; hence, the fraction of enzyme recoverable from $E_1$ is a function of both the ratio $k_2/(k_1+k_2)$ and the time of preincubation. The fraction of enzyme recoverable from $E_1$ ($f_{EU}$) at the end of various aging times could be obtained from the amplitude of the final fluorescence (corrected for time-dependent bleaching) produced upon quenching with DAPA and the use of the following relationship (Equation 20).
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FIG. 3. Dependence of the rate constant $k_{\text{obs}}$ on L-371,912 concentration under stopped-flow conditions. Thrombin (5–20 nM) was mixed with an equal volume of 23 μM Z-GPR-afc and L-371,912 (final concentration, 0.25–2 μM). The solid line represents the best fit of Equation 7 to the data.

![Graph](image)

FIG. 4. Dependence of the rate constants $k_{\text{obs}}(A)$ and $k_{\text{obs2}}(B)$ on L-370,518 concentration under stopped-flow conditions. Thrombin (2.5–50 nM) was mixed with an equal volume of 23 μM Z-GPR-afc and L-370,518 (final concentration, 0.025–1 μM). A, the solid line represents the best fit of Equation 9 to the data. The solid circles are the pseudo-first-order rate constants ($k_{\text{obs}}$) for displacement (as derived from stopped-flow) of 300 μM p-aminobenzenatidine from 0.5 μM thrombin by 2.5 and 5 μM L-370,518. To compare the different experimental procedures, the concentration of L-370,518 was adjusted to yield [L-370,518]$_{\text{act}}$ as described under "Experimental Procedures." B, the solid line represents the best nonlinear least squares fit of Equation 10 to the data.

The value of a plot of $k_{\text{obs}}$ against [L-370,518]$_{\text{act}}$ shows a sequential stopped-flow study of the interaction of the desketoamide L-371,912 with thrombin. DAPA was added to thrombin-L-371,912 that was premixed for increasing lengths of time (B–F, respectively). The traces of fluorescence versus time (after DAPA addition) were fit by Equation 11 to yield pseudo-first-order rate constants of 0.058, 0.053, 0.053, and 0.054 s$^{-1}$ for B–F, respectively. The mean value of $k_{-1}$, 0.054 s$^{-1}$, determined from the sequential stopped-flow experiment, is similar to the $k_{-1}$ value, 0.047 s$^{-1}$, determined from the relationship $k_{-1} = k_1 \times K_1$. Complete fluorescence recovery via a monophasic process that was independent of aging time with L-371,912 was additional evidence against the existence of a second complex ($E_2I$). Formation of significant $E_1I_2$ during the aging reaction (≥15 ms) would be expected to affect the recovery and/or rate of formation of uncomplexed $E$ (from $E_1I_1$ and $E_2I_2$) that is trapped with DAPA. As for L-370,518, a burst of fluorescence was observed with short aging times (Fig. 6B, traces C and S) suggesting incomplete conversion of $E + 1$ to $E_1I_1$. A plot of the log $f_{E_1I_1}$ (data not shown) versus aging time with the desketoamide L-371,912 yielded a pseudo-first-order rate constant of 18 s$^{-1}$. A second-order rate constant of 9 μM$^{-1}$ s$^{-1}$ was calculated from the equation $k_{-1} = 18$ s$^{-1}$/[L-371,912] using the experimental concentration of L-371,912 (2 μM). The $k_1$ value determined from sequential stopped-flow agreed with the $k_1$ values derived from the slopes in Figs. 3 and 5 (9.4 and 7.8 μM$^{-1}$ s$^{-1}$, respectively). Assuming $k_1 = 9.4$ μM$^{-1}$ s$^{-1}$ and $k_{-1} = 0.054$ s$^{-1}$, the kinetically determined equilibrium constant using Equation 4 is 5.7 nM which is similar to the equilibrium constant derived from inhibition of the steady state velocity for thrombin-catalyzed hydrolysis of a fluorogenic substrate (Fig. 1A).

Direct Determination of the Rate Constant for Dissociation of a Thrombin Inhibitor Complex—The value of $k_{-2}$ for dissociation of the thrombin-L-370,518 complex (Scheme II) was determined by preincubation of $E$ and $I$ (1:1.2 molar ratio) and subsequent dilution into substrate solution (at [S] $\gg K_m$). The progress curve showing the regeneration of enzyme activity was fit to Equation 11 and yielded a pseudo-first-order rate constant ($k_{-2}$) of $2 \times 10^{-4}$ s$^{-1}$ (data not shown). Since $k_{-1}$ is comparable to $k_{-2}$, $k_{-2}$ was calculated to be $3.6 \times 10^{-4}$ s$^{-1}$ using Equation 17 and the previously determined values for $k_{-2}$ ($2 \times 10^{-4}$ s$^{-1}$), $k_2$ (0.035 s$^{-1}$), and $k_{-1}$ (0.049 s$^{-1}$). A $K_c$ value was calculated as 136 pm, using Equation 5 and the experimentally determined values for $k_1$ (3.5 μM$^{-1}$ s$^{-1}$), $k_{-1}$ (0.049 s$^{-1}$), $k_2$
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FIG. 5. Dependence of the rate constant, \( k_{\text{obs}} \), for displacement of p-aminobenzamidine from the active site of thrombin on L-371,912 (squares) or L-370,518 (circles) concentration under stopped-flow conditions. Solutions containing thrombin (0.05–1 μM) and p-aminobenzamidine (100 μM) were mixed with L-371,912 (final concentration, 0.25–20 μM) or L-370,518 (final concentration, 2.5–50 μM) in the stopped-flow. The solid lines represent the best fit of Equation 7 to the data. Inset, monophasic loss of fluorescence from displacement of p-aminobenzamidine (50 μM) from the active site of thrombin (0.5 μM) by 5 μM L-371,912 (squares) or L-370,518 (circles). The solid lines represent the best nonlinear least squares fit of Equation 6 to the data and yielded \( k_{\text{obs}} \) values of 19.2 and 9.5 s\(^{-1}\) for L-371,912 and L-370,518, respectively.

(0.035 s\(^{-1}\)), and \( k_{-2} (3.4 \times 10^{-4} \text{ s}^{-1}) \). This estimate for \( K_i \) is reasonably close to 90 μM which was determined from the inhibitory effect of the ketoamide L-370,518 on the steady state velocity for thrombin-catalyzed hydrolysis of a fluorogenic substrate (Fig. 1B).

Structural Determinants of Thrombin Inhibitor Interactions—Substitution of a hydrogen atom for the N-terminal methylamino group in desketooamide L-371,912 and the ketoamide L-372,051, respectively (Table I). Like L-370,518 and L-371,912, L-372,011 was isolated as a single isomeric species. However, L-372,051 was composed of an equal mixture of an equal RS mixture at the a-carbon of 4-AChXGly, only one of the isomers was active as determined by thrombin titration (results not shown). The values of \( K_i \), \( K_i \text{ init} \), and \( k_1 \) for the ketoamide L-372,051 were adjusted for the concentration of the active isomer. L-372,011 and L-372,051 formed inhibitory complexes with thrombin with \( K_i \) values of 330 ± 30 nM and 4 ± 0.6 nM, respectively (Table II). The time-dependent inactivation of thrombin by the desketooamide L-372,011 (as derived from either substrate hydrolysis or p-aminobenzamidine displacement) was monophasic (data not shown). The linear dependence of the pseudo-first-order rate constant on L-372,011 concentration was fit by Equation 7, and the slope of the line yielded a \( k_1 \) value of 7 ± 0.5 μM\(^{-1}\) s\(^{-1}\) (data not shown). The \( k_1 \) value for the interaction between L-372,011 and thrombin was 2.4 s\(^{-1}\) as determined by sequential stopped-flow (data not shown). The monophasic time-dependent inhibition of thrombin, the linear dependence of the pseudo-first-order rate constant on the L-372,011 concentration, and the complete monophasic recovery of fluorescence in sequential stopped-flow studies are all consistent with L-372,011 inactivating thrombin via a one-step process. The ratio of \( k_{-1} (2.4 \text{ s}^{-1}) \) and \( k_1 (7 \mu \text{M}^{-1} \text{s}^{-1}) \) yielded a \( K_i \) value of 343 nM (Equation 4) for the thrombin-L-372,011 inhibitory complex. This \( K_i \) value based on kinetic rate constants agrees nicely with that determined (330 nM) from steady state measurements of L-372,011-mediated inhibition of thrombin-catalyzed substrate hydrolysis.

The time-dependent inactivation of thrombin by the ketoamide L-372,051 in the presence of substrate was a biphasic process that was fitted by Equation 16. The first (\( k_{\text{obs}1} \)) and second phase (\( k_{\text{obs}2} \)) exhibited a linear (Fig. 8A) and hyperbolic (Fig. 8B) dependence on the concentration of L-372,051, respectively. From a plot of \( k_{\text{obs}1} \text{ versus } L-372,051 \) concentration (Fig. 8B), values of 0.054 ± 0.002 s\(^{-1}\) and 285 ± 44 nM were determined for \( k_2 \) and \( K_i \text{ init} \), respectively (Equation 10). A plot of \( k_{\text{obs}2} \text{ versus } L-372,051 \) concentration (Fig. 8A) was analyzed using Equation 9 and yielded a \( k_1 \) value of 4.7 ± 0.5 μM\(^{-1}\) s\(^{-1}\).

The binding of the ketoamide L-372,051 to thrombin was also studied by p-aminobenzamidine displacement. In contrast to the behavior of the ketoamide L-370,518, displacement of p-aminobenzamidine from thrombin at low concentrations of L-372,051 was a biphasic process (data not shown). As expected the pseudo-first-order rate constants (\( k_{\text{obs}1} \) and \( k_{\text{obs}2} \)) derived from p-aminobenzamidine displacement were similar to those obtained from substrate hydrolysis (see Fig. 8). Monophasic and biphasic displacement of p-aminobenzamidine from thrombin obtained with the ketoamides L-370,518 and L-372,051, respectively, is due to their different \( K_i \text{ init} \) values. With L-370,518 concentrations used for the p-aminobenzamidine displacement experiment, residual free thrombin after transient formation of E1I (\( K_i \text{ init} = 15 \text{nM} \)) during the first phase was undetectable; in contrast, a substantial fraction of free thrombin was present at the end of the first phase with L-372,051 (\( K_i \text{ init} = 285 \text{nM} \)). When concentrations ([I] > 10 μM) of L-372,051 were much greater than \( K_i \text{ init} \) the second phase disappeared. The loss of the second phase at high L-372,051 concentrations is consistent with a two-step path-
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Fig. 6. Time-dependent increase of DAPA fluorescence using sequential stopped-flow. Thrombin (0.4 μM) was preincubated (first mix) with L-370,518 (2 μM) for 48 s (trace B), 140 s (trace C), 1 s (trace D), 19 s (trace E), and 192 s (trace F) (panel A) or with L-371,912 (2 μM) for 18 s (trace B), 48 s (trace C), 1 s (trace D), 19 s (trace E), and 192 s (trace F) (panel B) before final dilution (second mix) with 80 μM DAPA. Thrombin was also preincubated with DAPA for 3 s before dilution with L-370,518 (panel A, trace A) or L-371,912 (panel B, trace A). Only 3% of the collected data points are shown for reasons of clarity.

way (Scheme I). If the EI1 complex involving L-372,051 is in rapid equilibrium with E and I, an estimate for the $k_{-1}$ value, 1.34 s$^{-1}$, can be deduced from the relationship $k_{-1} = K_{i, init} \times k_1$ and the experimentally determined values of $K_{i, init}$ (285 μM) and $k_1$ (4.7 μM$^{-1}$ s$^{-1}$). Comparison of the $k_{-1}$ value with the value of $k_2$ (0.054 s$^{-1}$) obtained from Fig. 8B indicates that the assumption of a rapid equilibrium of EI1 with E and I ($k_{-1} \gg k_2$) is justified.

Fig. 9 shows the results of a sequential stopped-flow experiment wherein thrombin is reacted with L-372,051 for increasing lengths of time (traces B–H, respectively) and then reacted with DAPA. When the first reaction mixture is aged long enough to allow complete conversion of EI1 to EI2, no reaction with DAPA is observed (Fig. 9, trace H). The approach of fluorescence to its final value in traces B–G was a pseudo-first-order process (Equation 11) with a mean rate constant of 0.058 s$^{-1}$. Since $k_{-1} \gg k_2$, this rate constant should be equivalent to $k_{-1}$ (see Equation 18). As expected for a two-step process (when $k_{-1} \gg k_2$), the $k_{-1}$ value (1.34 s$^{-1}$) determined from the sequential stopped-flow experiment is equal to the value (1.34 s$^{-1}$) calculated from the relationship $k_{-1} = K_{i, init} \times k_1$. At short aging times (trace B, 42 ms), there was no significant burst of fluorescence suggesting that conversion of $E + I$ to EI1 was complete. This finding was anticipated since the L-372,051 concentration, 20 μM, was 10-fold higher than that used in the sequential stopped-flow experiment for L-370,518. Hence, the second-order rate constant ($k_1$) for association of L-372,051 with thrombin could not be determined in this experiment. At aging times <500 ms, all of the enzyme was recoverable from EI1. This observation is consistent with a two-step pathway, wherein $k_{-1} \gg k_2$ (i.e., EI1 dissociates to E and I more rapidly than it converts to EI2) and EI1 was not converted to EI2 (within the 500 ms time interval). At longer preincubation times (Fig. 9, traces C–H), EI1 was converted to EI2, hence, the fraction of enzyme recoverable from EI1 is a function of both $k_{-1}/(k_2 + k_{-1})$ and the preincubation time. The $f_{ER}$ at the end of various aging times was obtained as described for L-370,518. A log plot of $f_{ER}$ versus aging time yielded a pseudo-first-order rate constant of 0.058 s$^{-1}$ with an intercept at 1.0 (data not shown). One interpretation of the results is that 0.058 s$^{-1}$ is the pseudo-first-order rate constant for formation of EI2 from EI1, and 1.0 represents the fraction of enzyme recoverable ($k_{-1}/(k_{-1} + k_2)$) which is consistent with a two-step reaction scheme with $k_{-1} \gg k_2$. The $k_2$ value from sequential stopped-flow, 0.054 s$^{-1}$, is comparable to the $k_2$ value, 0.054 s$^{-1}$, from the limiting rate in a plot of $k_{obs}$ versus L-372,051 concentration (Fig. 8B).

The value of $k_{-2}$ for the reaction of the ketoamide L-372,051 with thrombin was determined as described previously for L-370,518 (data not shown). The progress curve depicting the regeneration of enzyme activity was fit by Equation 11 to yield a pseudo-first-order rate constant ($k_{off}$) of $8 \times 10^{-4}$ s$^{-1}$ (data not shown). Since $k_{-1} \gg k_2$, Equation 17 indicates that the $k_{off}$ value (8 × 10$^{-4}$ s$^{-1}$) is essentially equivalent to $k_{-2}$. Using Equation 5 and the experimentally determined values of $k_2$ (4.72 μM$^{-1}$ s$^{-1}$), $k_{-1}$ (1.34 s$^{-1}$), $k_2$ (0.054 s$^{-1}$), and $k_{-2}$ (8 × 10$^{-4}$ s$^{-1}$)
a value of 4.2 nm was calculated for \( K_i \). The calculated value of \( K_i \) (4.2 nm) agreed with the value determined (4 nM) from steady state measurements of the inhibition of thrombin-catalyzed substrate hydrolysis by L-372,051.

Replacement of the P1
\(_9\) N-methylcarboxamide in L-370,518 with azetidylcarboxamide yielded ketoamide L-372,228 (Table I). Using methods previously described for L-370,518 and L-372,051, the equilibrium and rate constants for the inactivation of thrombin by L-372,228 were determined (Table II). The equilibrium constant for the inhibition of thrombin-catalyzed hydrolysis of a fluorogenic substrate by the azetidyl-substituted ketoamide L-372,228 was 40 \( \times \) 5 pm. The kinetically determined equilibrium constant for the thrombin-L-372,228 complex using Equation 5 and the experimentally determined values of \( k_1 \) (0.52 \( \mu \)M\(^{-1}\) s\(^{-1}\)), \( k_{-1} \) (0.42 s\(^{-1}\)), \( k_2 \) (0.56 s\(^{-1}\)), and \( k_{-2} \) (3.5 \( \times \) 10\(^{-5}\) s\(^{-1}\)) was 50 pm which was similar to the value of 40 pm derived from steady state measurements of the inhibition of thrombin-catalyzed substrate hydrolysis by L-372,228.

DISCUSSION
The sequential stopped-flow procedures used in this study enabled us for the first time to determine directly the individual rate constants \( k_1, k_{-1}, \) and \( k_2 \) for a two-step inhibitory pathway without invoking any assumptions regarding the relative rates of the first and second steps. Prior evaluation of individual rate constants of two-step inhibitory reactions where \( k_{-1} \) was comparable to \( k_2 \) (e.g. Ref. 22), have relied upon the effects of viscosogens on reaction rates with the assumption that the viscosity enhancing agents only alters diffusion-con-
trolled processes and does not compromise the conformation of the reactants. Our sequential stopped-flow method avoided the use of perturbants and allowed us to directly and precisely evaluate the individual rate constants for two-step inhibitory pathways.

We have presented evidence that the desketoamides, L-371,912 and L-372,011, inhibit thrombin via a one-step pathway; in contrast, their respective α-ketoamide analogs, L-370,518 and L-372,051, inhibit thrombin via a two-step pathway. It is unlikely that the two-step binding pathway seen with the ketoamides reflects a cis to trans conformational transition around the proline amide bond of the inhibitor, since this proline amide bond is in a trans conformation in both the desketoamide L-371,912 and the corresponding ketoamide L-370,518 complexes of thrombin (12). The value of the equilibrium constant ($K_{-1} = 14 \text{ nM}$) for dissociation of the $EI_1$ complexes of these inhibitors cannot be determined.
complex between thrombin and L-370,518 was similar to the value of the equilibrium constant ($K_1 = 5 \text{ nM}$) for dissociation of the thrombin-L-371,912 complex. Additionally, similar values were observed for the equilibrium constant ($K_2 = 285 \text{ nM}$) for dissociation of the initial complex between thrombin and L-372,051 and the dissociation constant for the thrombin-L-372,011 complex ($K_3 = 330 \text{ nM}$). This correspondence is consistent with the view that L-370,518 and L-372,051 form initial complexes with thrombin wherein the $\alpha$-ketoamide moiety interacts minimally with thrombin, and the interactions between thrombin and the remainder of the inhibitors are similar to those of the corresponding desketoamide analogs. If this view is correct, the second step in the inhibitory pathway observed with the $\alpha$-ketoamide inhibitors would reflect formation of the hemiketal adduct, together with any reorientation of the inhibitor dictated by the geometrical constraints imposed by the hemiketal formation.

Substitution of an $\alpha$-ketoamide group for the hydrogen atom in L-371,912 ($K_1 = 5 \text{ nM}$) to yield L-370,518 ($K_2 = 90 \text{ pm}$) resulted in only a 56-fold gain in inhibitory potency (10-12), whereas the same substitution in L-372,112 (Table II) ($K_3 = 1.2 \text{ uM}$) to afford L-373,310 ($K_4 = 2.8 \text{ nM}$) resulted in a 429-fold gain in inhibitory potency (8, 9). Interestingly, the cyclohexyl group of L-370,518 in the thrombin inhibitor complex is rotated 90° from its position in the thrombin-L-371,912 complex (12). This fact suggests that formation of the hemiketal adduct of L-370,518 with thrombin may force the cyclohexylamino group to assume a conformation in the S1 pocket that is energetically less favorable than that in the thrombin-L-371,912 complex, wherein the cyclohexylamino group has a greater degree of freedom to optimize its interaction in the S1 pocket. In contrast, formation of the corresponding hemiketal adduct of L-370,310 with thrombin may not result in less favorable binding interactions between the less bulky e-amino alkyl chain and the S1 pocket in thrombin. These factors may well account for the fact that a lower potency ratio is observed for the L-370,518/L-371,912 ketoamide/desketoamide pair than is observed for the corresponding L-370,310/L-372,112 ketoamide/desketoamide pair.

The observation that L-372,228 forms a weaker initial complex with thrombin than does L-370,518 suggests that the more bulky P1' group in L-372,228 contributes repulsive interactions with thrombin in the initial inhibitory complex. Surprisingly, these unfavorable interactions appear to be restricted to the EI1 complex as evidenced by the 16-fold increase in the value of $k_2$ and the 10-fold decrease of $k_{-2}$ for the binding of L-372,228 to thrombin relative to the corresponding kinetic constants for L-370,518. This change from an endergonic contribution of the P1' group of L-372,228 in the EI1 complex to an exergonic contribution in the EI1 complex is consistent with reorientation of thrombin and/or inhibitor upon formation of the hemiketal adduct involving the active site serine residue of thrombin.

The enhanced inhibitory potency of L-370,518 relative to that of L-372,051 reflects the importance of the hydrogen bond between the P3 amino group of L-370,518 and Gly-2162 of thrombin as shown by x-ray crystallographic analysis (12). Moreover, the increased stability of the initial complex formed with L-370,518 relative to that of the initial complex formed with L-372,051 suggests that the P3 amino hydrogen bond is formed in the initial complex. Comparisons of rate constants for formation and dissociation of the EI1 complexes formed with L-370,518 and L-372,051 indicate that the predominant effect of this hydrogen bond is to decrease the rate constant for dissociation of the initial complex by about 27-fold (Table II).

In summary we have demonstrated that certain $\alpha$-ketoamide inhibitors of thrombin inactivate thrombin via a two-step pathway, wherein formation of the covalent bond between the inhibitor and the active site serine residue probably occurs in the second step. Additionally, individual rate constants for the two-step inhibitory pathway were evaluated directly using a novel sequential stopped-flow analysis which should be applicable to the kinetic characterization of other enzyme-inhibitor pathways.

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Characterization of the Two-step Pathway for Inhibition of Thrombin by α-Ketoamide Transition State Analogs

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