Y., & Davie, E. W. (1987) *Biochemistry 26*, 7003-7011.
Fry, D. C., Madison, V. S., Bolin, D. R., Greeley, D. N., Toome, V., & Wegrzynski, B. B. (1989) *Biochemistry 28*, 2399-2409.
Furie, B., & Furie, B. C. (1988) *Cell 53*, 505-518.
Green, F. R., Lynch, B., & Kaiser, E. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8340-8344.
Hubbard, B., Jacobs, M., Ulrich, M., Walsh, C., Furie, B., & Furie, B. C. (1989a) *J. Biol. Chem. 264*, 14145-14150.
Hubbard, B. R., Ulrich, M. M. W., Jacobs, M., Vermeer, C., Walsh, C., Furie, B., & Furie, B. C. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6893-6897.
Huber, P., Schmitz, T., Griffin, J., Jacobs, M., Walsh, C., Furie, B., & Furie, B. C. (1990) *J. Biol. Chem. 265*, 12467-12473.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R., (1979) *J. Chem. Phys. 71*, 4546-4553.
Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., & Furie, B. (1987) *Cell 48*, 185-191.
Karplus, M. (1959) *J. Phys. Chem. 30*, 11-15.
Marion, D., & Wuthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967-974.
Merrifield, R. B. (1965) *Science 150*, 178-185.
Otting, G., Widmer, H., Wagner, G., & Wüthrich, K. (1986) *J. Magn. Reson. 66*, 187-193.
Pan, L. C., & Price, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6109-6113.
Plateau, P., & Guéron, M. (1982) *J. Am. Chem. Soc. 104*, 7310-7311.
Price, P. A., & Williamson, M. K. (1985) *J. Biol. Chem. 260*, 14971-14975.
Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479-485.
Taniuchi, H., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 3864-3875.
Ulrich, M. M. W., Furie, B., Jacobs, M. R., Vermeer, C., & Furie, B. C. (1988) *J. Biol. Chem. 263*, 9697-9702.
Ware, J., Diuguid, D. L., Liebman, H. A., Rabiet, M.-J., Kasper, C. K., Furie, B. C., Furie, B., & Stafford, D. W. (1989) *J. Biol. Chem. 264*, 11401-11406.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Wright, P. E., Dyson, H. J., & Lerner, R. A. (1988) *Biochemistry 27*, 7167-7175.

Mechanistic Studies on Thrombin Catalysis

Stuart R. Stone,* Andreas Betz, and Jan Hofsteenge
Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland
Received May 13, 1991; Revised Manuscript Received July 23, 1991

ABSTRACT: The kinetic mechanism of the cleavage of four *p*-nitroanilide (pNA) substrates by human α-thrombin has been investigated by using a number of steady-state kinetic techniques. Solvent isotope and viscosity effects were used to determine the stickiness of the substrates at the pH optimum of the reaction; a sticky substrate is defined as one that undergoes catalysis faster than it dissociates from the Michaelis complex. Whereas benzoyl-Arg-pNA could be classified as a nonsticky substrate, D-Phe-pipecolyl-Arg-pNA was very sticky. The other two substrates (tosyl-Gly-Pro-Arg-pNA and acetyl-D-Phe-pipecolyl-Arg-pNA) were slightly sticky. The pH profiles of k_{cat}/K_m were bell-shaped for all substrates. The pK_a values determined from the pH dependence of k_{cat}/K_m for benzoyl-Arg-pNA were about 7.5 and 9.1. Similar pK_a values were determined from the pH profiles of k_{cat}/K_m for tosyl-Gly-Pro-Arg-pNA and acetyl-D-Phe-pipecolyl-Arg-pNA and for the binding of the competitive inhibitor N^{α}-dansyl-L-arginine-4-methylpiperidine amide. The groups responsible for the observed pK_a values were proposed to be His57 and the α-amino group of Ile16. The temperature dependence of the pK_a values was consistent with this assignment. The pK_a values of 6.7 and 8.6 observed in the pH profile of k_{cat}/K_m for D-Phe-pipecolyl-Arg-pNA were displaced to lower values than those observed for the other substrates. The displacement of the acidic pK_a value could be attributed to the stickiness of this substrate. The basic pK_a value of 8.6 was caused by preferential binding of substrate molecules with protonated α-amino groups. The pH dependence of k_{cat} for benzoyl-Arg-pNA was consistent with the binding of the substrate decreasing the pK_a of His57 by more than 1 unit. The pH dependence of k_{cat} for the other three substrates was more complicated and suggested that the rate of catalysis was influenced by the ionization of groups not directly involved in catalysis.

Thrombin is a serine protease with considerable homology to chymotrypsin in trypsin in terms of both primary and tertiary structure (Bode et al., 1989). Like trypsin, thrombin shows a preference for cleaving substrates C-terminal to a basic residue, but the substrate specificity of thrombin is more restricted than that of trypsin (Chang, 1986; Lottenberg et al., 1981). In addition, thrombin appears to use sites that are distant from the active site in order to achieve specific interactions with macromolecular substrates, cofactors, and inhibitors (Fenton, 1981; Fenton & Bing, 1986). The recently determined crystal structures of D-Phe-Pro-ArgCH_2-thrombin (Bode et al., 1989) and hirudin-thrombin complexes (Rydel

* Correspondence should be addressed to this author at his present address: Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

0006-2960/91/0430-9841$02.50/0

et al., 1990; Grütter et al., 1990) provide some idea of the structural basis for thrombin's specificity. An insertion loop (Tyr60A–Trp60D)[1] of thrombin restricts the active site and this probably explains why so few macromolecular substrates and inhibitors of serine proteases bind well to thrombin. In addition, hydrophobic regions within the active-site cleft would appear to be important for the binding of substrates and inhibitors (Bode et al., 1989). The inhibitor hirudin also uses binding sites distant from the catalytic center. The C-terminal region of hirudin is bound to a surface groove that has been termed the anion-binding exosite, and it seems likely that this region of thrombin also interacts with the substrate fibrinogen and the cell-surface protein thrombomodulin (Rydel et al., 1990; Grütter et al., 1990).

The kinetic mechanism of cleavage of *p*-nitroanilide substrates by thrombin has not been extensively investigated. The careful studies by Lottenberg et al. (1981, 1983) have documented the substrate specificity of the enzyme, and a recent study has investigated the pH and salt dependence of the kinetic parameters (De Cristofaro & Di Cera, 1990). However, a number of points remain to be established; for example, which catalytic step is rate limiting in the cleavage of *p*-nitroanilide substrate (acylation or deacylation) and to what extent the binding step preceding the catalytic step comes to equilibrium with different substrates. In the present paper, we report the results of investigations designed to answer such questions. A range of steady-state kinetic techniques, including the study of viscosity, solvent isotope, and pH effects, has been used to determine the kinetic mechanism of the thrombin-catalyzed cleavage of four *p*-nitroanilide substrates.

Experimental Procedures

Materials. The substrates Bz-Arg-pNA,[2] tos-Gly-Pro-Arg-pNA (Chromozym TH), and D-Phe-Pip-Arg-pNA (S-2238) were obtained from Bachem (Bubendorf, Switzerland), Boehringer-Mannheim (Mannheim, Germany) and Kabi-Vitrum (Molndal, Sweden), respectively. Ac-D-Phe-Pip-Arg-pNA was prepared from D-Phe-Pip-Arg-pNA by acetylation with *p*-nitrophenyl acetate followed by purification by reversed phase HPLC with a C_{18} column. Human α-thrombin was prepared and characterized as described previously (Stone & Hofsteenge, 1986). The preparation used was fully active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973) and was essentially free from degraded forms as assessed by gel electrophoresis and Edman degradation.

Synthesis of N^{α}-Dansyl-L-arginine-4-methylpiperidine Amide (DAMPA). The mixed anhydride of *N-t*-Boc-di-Cbz-L-arginine (3.68 mmol) was prepared by standard methods and reacted with 4-methylpiperidine (4.78 mmol). The neutral fraction was purified by chromatography on a column of silica gel and the *t*-Boc protecting group was removed by reaction with trifluoroacetic acid. The product (3.02 g) was reacted with dansyl chloride (4.39 mmol) for 4 h at 0 °C in 15 mL of chloroform containing 11 mmol of triethylamine. The neutral dansylated product was purified on a column of silica. The Cbz groups were removed by reduction in 30 mL of methanol containing 2.5 mL of acetic acid, 340 mg of ammonium formate and 150 mg of Pd (10%) on active carbon. The catalyst was removed by filtration and the product was recovered by lyophilization. Yield: 1.1 g (49%). The final product gave one spot (R_f = 0.58) on TLC with butanol–acetic acid–water (3:1:1) which was fluorescent under long-wavelength UV light. The absorption spectrum showed maxima at 246 and 330 nm.

Amidolytic assays were performed in a three-buffer system containing 0.1 M MES, 0.05 M Tris, 0.05 M ethanolamine, 0.2 M NaCl, 0.2% (w/v) poly(ethylene glycol) of M_r 6000, and substrate. The pH of the assay buffer was adjusted to the desired pH at about the temperature of the assay. The precise pH of the assay was measured later at the temperature used with a Radiometer PHM83 pH meter. In experiments in which assays were conducted in D_2O, the pD was taken to equal the pH meter reading plus 0.4 (Fersht, 1985). The three-buffer system was chosen because it provided adequate buffering capacity over the pH range used (pH 6–10) and the ionic strength varied only slightly over this range (Ellis & Morrison, 1982).

The release of *p*-nitroaniline due to cleavage of the substrates was monitored at 400–410 nm by using a Hewlett-Packard 8452A spectrophotometer. The extinction change for the reaction under the conditions of the assay was determined to be 9300 M^{-1} cm^{-1}. The concentrations of the substrates were determined from their absorbance at 342 nm as described by Lottenberg and Jackson (1983).

For the determination of k_{cat} and K_m values, six different substrate concentrations that varied over an 11-fold range were used. Dilutions of substrate were made as recommended by Fromm (1975), and at least one concentration was above and one below the K_m value. At pH values above 9, a significant nonenzymatic rate of hydrolysis of the substrates tos-Gly-Pro-Arg-pNA and Bz-Arg-pNA was observed and this rate was subtracted from the enzyme-catalyzed rate. The concentration of thrombin used was chosen such that measurable initial rates would be obtained and less than 10% of the substrate would be utilized in the first 5 min of the assay. The concentration of thrombin used varied from 10 pM for assays with D-Phe-Pip-Arg-pNA to 0.2 μM for those with Bz-Arg-pNA. The inhibition of thrombin by the substrate analogue DAMPA was studied in the presence of 100 μM D-Phe-Pip-Arg-pNA and with 50 pM enzyme.

For experiments examining the effect of viscosity on the kinetic parameters, the viscosity of the assay was determined at 37 °C by using an Ubbelodhe viscometer.

Theory and Data Analysis

The reaction catalyzed by thrombin can be represented by Scheme I, where E is the enzyme, S is the substrate, ES is the Michaelis complex, EP_2 is the acyl–enzyme complex, and P_1 and P_2 are the hydrolysis products [*p*-nitroaniline and peptide (or Bz-ArgOH), respectively].

Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightharpoonup} P_1 + EP_2 \overset{k_3}{\rightharpoonup} E + P_2$$

Under steady-state conditions, the dependence of velocity of the reaction (v) on the substrate concentration can be described by the Michaelis–Menten equation:

$$\frac{v}{[E_0]} = \frac{k_{cat}[S]}{K_m + [S]} \quad (1)$$

where $[E_0]$ is the enzyme concentration. For the reaction mechanism presented in Scheme I, k_{cat} (catalytic constant) and K_m (Michaelis constant) can be defined by eqs 2a and 3a. For *p*-nitroanilide substrates, it is generally accepted that

[1] The sequence numbering for thrombin is that of Bode et al. (1989) and is based on chymotrypsin numbering.

[2] Abbreviations: Ac, acetyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; DAMPA, N^{α}-dansyl-L-arginine-4-methylpiperidine amide; Pip, pipecolyl; pNA, *p*-nitroanilide; *t*-Boc, *tert*-butyloxycarbonyl; tos, 4-toluenesulfonyl.

acylation is rate limiting ($k_3 \gg k_2$), and if this is also the case for thrombin, the expressions for k_{cat} and K_m can be simplified to eqs 2b and 3b. If deacylation is rate limiting ($k_2 \gg k_3$), the expressions for k_{cat} and K_m are given by eqs 2c and 3c (Polgár, 1987).

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (2a)$$

$$\text{If } k_3 \gg k_2, \text{ then } k_{cat} = k_2 \quad (2b)$$

$$\text{If } k_2 \gg k_3, \text{ then } k_{cat} = k_3 \quad (2c)$$

$$K_m = [(k_{-1} + k_2)k_3]/[k_1(k_2 + k_3)] \quad (3a)$$

$$\text{If } k_3 \gg k_2, \text{ then } K_m = (k_{-1} + k_2)/k_1 \quad (3b)$$

$$\text{If } k_2 \gg k_3, \text{ then } K_m = [(k_{-1} + k_2)k_3]/k_1k_2 \quad (3c)$$

Viscosity Effects. A sticky substrate is defined as one that dissociates from the Michaelis complex more slowly than this complex undergoes catalysis, i.e., $k_2 > k_{-1}$ (Cleland, 1977). For a nonsticky substrate, dissociation from the Michaelis complex is faster than reaction ($k_{-1} > k_2$) and the binding step comes to equilibrium. The stickiness of a substrate can be determined from the effect of viscosity on k_{cat}/K_m (Brouwer & Kirsch, 1982; Cleland, 1986). For the mechanism presented in Scheme I, the value of k_{cat}/K_m will be given by

$$\frac{k_{cat}}{K_m} = \frac{k_1k_2}{k_{-1} + k_2} \quad (4)$$

This equation applies irrespective of whether acylation ($k_2 \ll k_3$) or deacylation ($k_2 \gg k_3$) is rate limiting. The magnitudes of both k_1 and k_{-1} will be dependent on the viscosity of the assay and it has been shown that the dependence of k_{cat}/K_m on the relative viscosity of the solution (η_{rel}) is given by (Brouwer & Kirsch, 1982)

$$\frac{k_{cat}}{K_m} = \frac{k_1k_2/\eta_{rel}}{k_{-1}/\eta_{rel} + k_2} \quad (5)$$

where η_{rel} is the viscosity of the assay with added viscogenic reagent relative to that of the assay without any such reagent. When eq 5 is written in the double-reciprocal form (eq 6), it is apparent that a plot of K_m/k_{cat} against η_{rel} will be linear with a slope of $1/k_1$ and a horizontal intercept of $-k_{-1}/k_2$:

$$\frac{K_m}{k_{cat}} = \frac{\eta_{rel}}{k_1} + \frac{k_{-1}}{k_1k_2} \quad (6)$$

Initial velocity data obtained by varying the substrate at a particular viscosity were fitted to eq 1 by using weighted, robust linear regression (Cornish-Bowden & Endrenyi, 1981). The resulting values of k_{cat}/K_m were weighted according to the inverse of their variances and fitted to eq 6 to obtain estimates for k_1 and k_2/k_{-1}.

Solvent Isotope Effects. The general base catalysis involving the catalytic histidine of thrombin is expected to proceed more slowly in D_2O (Polgár, 1987). If acylation is rate limiting ($k_2 \ll k_3$, Scheme I), the observed solvent isotope effects on k_{cat} ($^Dk_{cat}$) and k_{cat}/K_m [$^D(k_{cat}/K_m)$] will be given by

$$^Dk_{cat} = k_2/^Dk_2 \quad (7)$$

$$^D(k_{cat}/K_m) = \frac{k_2(k_{-1} + {}^Dk_2)}{{}^Dk_2(k_{-1} + k_2)} \quad (8)$$

where $^Dk_2$ is the rate of acylation in the presence of D_2O. When the substrate is not sticky ($k_{-1} \gg k_2$), $^D(k_{cat}/K_m)$ will equal $^Dk_{cat}$. In contrast, there will be no solvent isotope effect on k_{cat}/K_m for a sticky substrate ($k_{-1} \ll k_2$), i.e., $^D(k_{cat}/K_m)$ will equal 1.0.

If deacylation is rate limiting ($k_2 \gg k_3$), $^Dk_{cat}$ will be given by eq 9, while eq 8 will still describe $^D(k_{cat}/K_m)$:

$$^Dk_{cat} = k_3/^Dk_3 \quad (9)$$

In this case, the value of $^D(k_{cat}/K_m)$ will equal 1.0 for a sticky substrate and it will be significantly greater than 1.0 but will not necessarily equal $^Dk_{cat}$ when the substrate is not sticky.

pH Dependence of the Interaction with Substrates and Inhibitors. The pH profiles of k_{cat}/K_m for *p*-nitroanilide substrates with thrombin were bell-shaped and could be described by

$$\frac{k_{cat}}{K_m} = \frac{k_{cat}/K_m(opt)}{1 + [H]/K_1 + K_2/[H]} \quad (10)$$

where $k_{cat}/K_m(opt)$ is the pH-independent value of k_{cat}/K_m and K_1 and K_2 are acid dissociation constants. Initial-velocity data for substrates obtained at a particular pH were fitted to eq 1 as described above to obtain estimates for k_{cat}, K_m, and k_{cat}/K_m. The values of k_{cat}/K_m obtained from these analyses were weighted according to the inverse of their variances and fitted to eq 10 by weighted, nonlinear regression in order to obtain estimates for pK_1 and pK_2.

Estimates for the enthalpy of ionization (ΔH_{ion}) of the groups responsible for the observed pK_a values can be obtained from a plot of the pK_a values against the reciprocal of absolute temperature ($1/T$); the slope of such a plot is $\Delta H_{ion}/2.303R$, where R is the gas constant (Cleland, 1977; Tipton & Dixon, 1979). The slope of the plot of the pK_a values against $1/T$ was determined from data obtained at three temperatures with tos-Gly-Pro-Arg-pNA by weighting the estimates of pK_a values according to the inverse of their variances and using weighted linear regression. For D-Phe-Pip-Arg-pNA, pK_a values were only obtained at two temperatures and estimates of ΔH_{ion} for the groups responsible were calculated by using the formula $\Delta H_{ion} = 2.303R\Delta pK_a/\Delta(1/T)$ (Tipton & Dixon, 1979).

The pH dependence of k_{cat} was in general more complicated than that observed for k_{cat}/K_m. A simple half-bell-shaped profile that could be described by eq 11 was, however, observed with Bz-Arg-pNA:

$$k_{cat} = \frac{k_{cat}(opt)}{1 + [H]/K_1} \quad (11)$$

where $k_{cat}(opt)$ is the pH-independent value of k_{cat}. Estimates of k_{cat} determined at different pH values were weighted according to the inverse of their variances and fitted to eq 11 by weighted, nonlinear regression in order to obtain an estimate for pK_1.

The pH dependence of the dissociation constant (K_i) for the competitive inhibitor DAMPA exhibited a bell-shaped profile that could be described by

$$K_i = K_i(opt)(1 + [H]/K_1 + K_2/[H]) \quad (12)$$

where $K_i(opt)$ is the pH-independent value of K_i. Initial-velocity data for the inhibition of thrombin by the competitive inhibitor DAMPA were fitted to the Dixon equation by using weighted, robust linear regression (Cornish-Bowden & Endrenyi, 1981) to yield estimates of the apparent dissociation constant ($K_{i'}$) at each pH. Estimates for K_i were calculated by using the following relationship together with the concentration of substrate and its K_m value at the particular pH:

$$K_i = \frac{K_{i'}}{1 + [S]/K_m} \quad (13)$$

The values of K_i were weighted according to their inverse

Table I: pK_a and pH-Independent Values Derived from the Variation of k_{cat}/K_m for *p*-Nitroanilide Substrates[a]

substrate	pH-independent value (M^{-1} s^{-1})	pK_1	pK_2
37 °C			
Bz-Arg-pNA	(1.34 ± 0.07) × 10^3	7.57 ± 0.03	9.09 ± 0.05
tos-Gly-Pro-Arg-pNA	(3.34 ± 0.09) × 10^7	7.28 ± 0.02	9.10 ± 0.03
D-Phe-Pip-Arg-pNA	(8.62 ± 0.37) × 10^7	6.71 ± 0.03	8.63 ± 0.03
Ac-D-Phe-Pip-Arg-pNA	(2.30 ± 0.04) × 10^6	7.30 ± 0.03	9.15 ± 0.05
25 °C			
tos-Gly-Pro-Arg-pNA	(4.34 ± 0.29) × 10^7	7.64 ± 0.05	9.31 ± 0.06
15 °C			
tos-Gly-Pro-Arg-pNA	(2.98 ± 0.13) × 10^7	7.57 ± 0.02	9.70 ± 0.07
D-Phe-Pip-Arg-pNA	(4.77 ± 0.32) × 10^7	6.94 ± 0.04	9.43 ± 0.06

[a] Estimates for k_{cat}/K_m were determined between pH 6 and 10 as described under Experimental Procedures. These values were fitted by weighted nonlinear regression to eq 10 to obtain the estimates of the parameters, which are given together with their standard errors.

variance and fitted by linear regression to eq 12 in order to obtain estimates of pK_1 and pK_2 for the inhibition by DAMPA.

Results

pH Dependence of k_{cat}/K_m. Values for k_{cat}/K_m and k_{cat} were determined with four substrates over the pH range 6.0–10.0 at 37 °C. The ionic strength was maintained constant at 0.3 M by using a three-component buffer mixture as recommended by Ellis and Morrison (1982). At this ionic strength, thrombin was stable over the entire pH range for at least 30 min. At the lower ionic strength of 0.15 M, a slight loss of thrombin activity was detected over time periods greater than 20 min. The pH profile of k_{cat}/K_m for each of the substrates examined was bell-shaped (Figure 1). However, the pK_a values determined from the profiles varied. For the poor substrate Bz-Arg-pNA, pK_a values of 7.57 ± 0.03 and 9.09 ± 0.05 were observed (Table I). Similar pK_a values (7.28 ± 0.02 and 9.10 ± 0.03) were obtained for tos-Gly-Pro-Arg-pNA (Chromozym TH). In contrast, analysis of the pH dependence of k_{cat}/K_m for D-Phe-Pip-Arg-pNA (S-2238) yielded pK_a values (6.71 ± 0.03 and 8.63 ± 0.03) that were displaced in relation to those obtained for the above two substrates. This displacement was not seen when the α-amino group of D-Phe-Pip-Arg-pNA was acetylated; the pK_a values obtained from the pH dependence of k_{cat}/K_m for Ac-D-Phe-Pip-Arg-pNA were 7.30 ± 0.03 and 9.15 ± 0.05 (Table I).

The pH dependence of k_{cat}/K_m for tos-Gly-Pro-Arg-pNA was also investigated at 15 and 25 °C (data not shown). The determined pK_a values were higher at the lower temperatures (Table I). The temperature dependence of the pK_a values was analyzed as described under Theory and Data Analysis to yield estimates of ΔH_{ion} for the groups involved. Values of 22 ± 8 and 43 ± 7 kJ mol^{-1} were determined for the ΔH_{ion} of the groups responsible for pK_1 and pK_2, respectively. These values of ΔH_{ion} are consistent with a histidine or sulfhydryl being responsible for pK_1 and a primary amino group for pK_2 (Tipton & Dixon, 1979). A sulfhydryl group can be excluded for pK_1 since thrombin contains no free cysteines.

The pH dependence of k_{cat}/K_m for D-Phe-Pip-Arg-pNA was also determined at 15 °C and values of 6.94 ± 0.04 and 9.43 ± 0.06 were obtained for pK_1 and pK_2, respectively (Table I). Estimates for ΔH_{ion} were determined from the changes in the pK_a values as described under Theory and Data Analysis. The values of ΔH_{ion} of 18 and 62 kJ mol^{-1} derived from the change in pK_1 and pK_2, respectively, are also consistent with a histidine being responsible for pK_1 and a primary amino group for pK_2.

pH Dependence of k_{cat} *for p-Nitroanilide Substrates.* In contrast to the normal bell-shaped pH profiles for k_{cat}/K_m, the pH dependence of k_{cat} was in general more complex (Figure 2). A simple pH dependence of k_{cat} was seen only in the case of Bz-Arg-pNA, where catalytic activity appeared to depend on the ionized form of a group with a pK_a value of 6.34 ± 0.04.

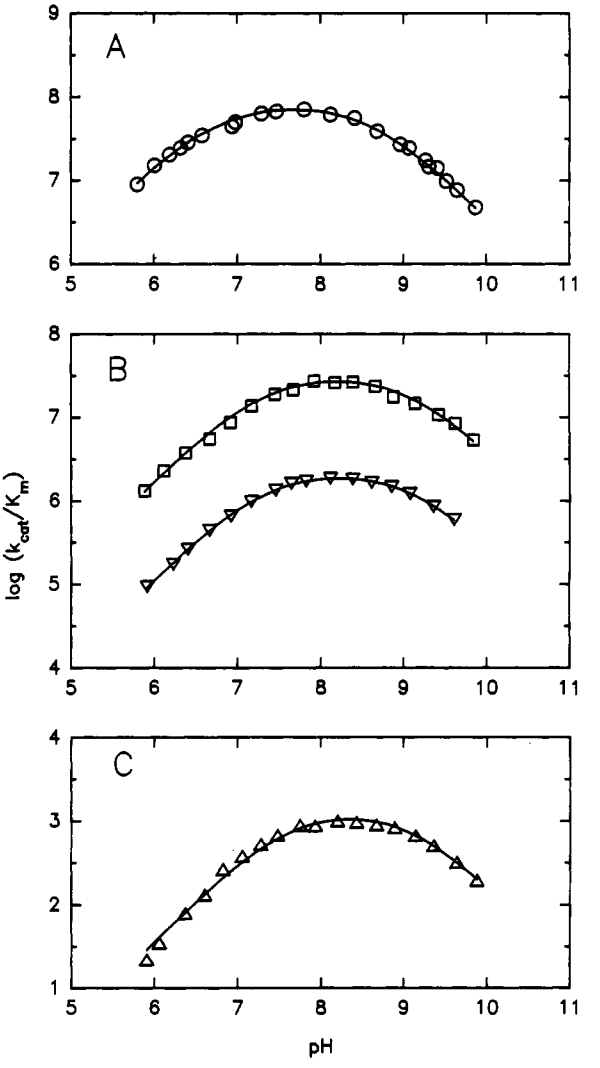


FIGURE 1: Variation with pH of log(k_{cat}/K_m) for the cleavage of *p*-nitroanilide substrates by thrombin. The units of k_{cat}/K_m are M^{-1} s^{-1} and the values are plotted for (A) D-Phe-Pip-Arg-pNA (○), (B) Ac-D-Phe-Pip-Arg-pNA (▽), tos-Gly-Pro-Arg-pNA (□), and (C) Bz-Arg-pNA (△). The curves represent the fit of the data to eq 10 and were drawn using the values of the parameters given in Table I.

The pH-independent value of k_{cat} was 0.186 ± 0.003 s^{-1}. The value of k_{cat} for tos-Gly-Pro-Arg-pNA increased gradually between pH 6 and 9 (Figure 2). A similar pH dependence of k_{cat} for tos-Gly-Pro-Arg-pNA was also seen at 15 and 25 °C (data not shown). The variation of k_{cat} for D-Phe-Pip-Arg-pNA and for Ac-D-Phe-Pip-Arg-pNA with pH at 37 °C did not reflect the ionization states of any groups that were essential for catalysis (Figure 2). The value of k_{cat} for these two substrates was relatively constant over the pH range tested.

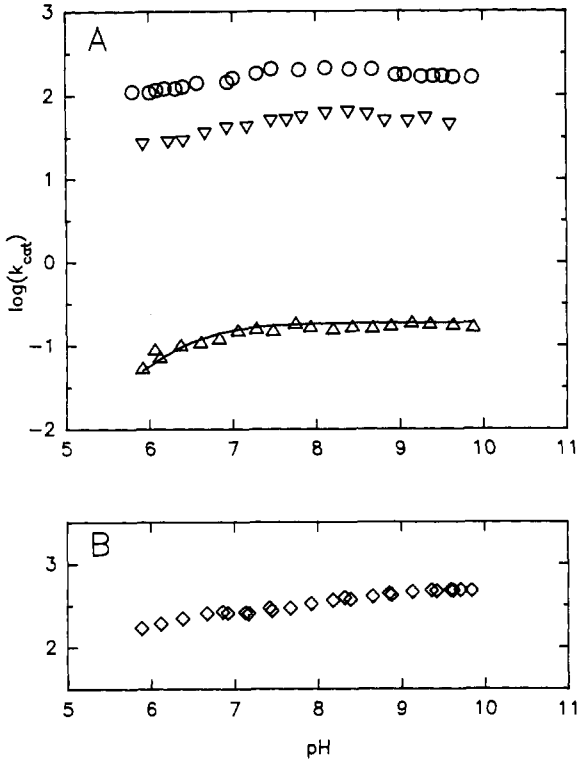


FIGURE 2: Variation with pH of log(k_{cat}) for the cleavage of *p*-nitroanilide substrates by thrombin. The units of k_{cat} are s^{-1} and values are plotted for (A) Bz-Arg-pNA (△), Ac-D-Phe-Pip-Arg-pNA (▽), D-Phe-Pip-Arg-pNA (○), and (B) tos-Gly-Pro-Arg-pNA (◇). The curve drawn for Bz-Arg-pNA represents the fit of the data to eq 11.

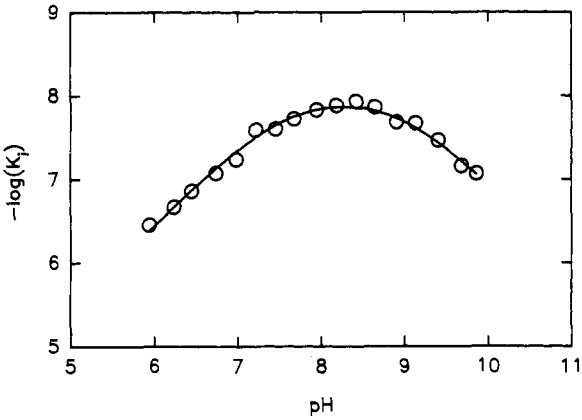


FIGURE 3: Variation with pH of log(K_i) for DAMPA. The units of K_i are M and the curve drawn represents the fit of the data to eq 12.

Optimal catalytic activity was observed between pH 7.5 and 8.5; a small decrease in k_{cat} of 2-fold or less was seen at pH values above 8.5 and below 7.5 (Figure 2). A similar pH dependence of k_{cat} for D-Phe-Pip-Arg-pNA was also seen at 15 °C (data not shown).

pH Dependence of Inhibition by DAMPA. The pH dependence of the dissociation constant (K_i) for the substrate analogue DAMPA exhibited a bell-shaped profile as shown in Figure 3. Analysis of these data yielded values of 7.56 ± 0.06 and 8.96 ± 0.08 for pK_1 and pK_2, respectively. The pH-independent value of K_i was 9.7 ± 1.2 nM.

Viscosity Effects. The effect of the viscosity on the reaction catalyzed by thrombin has been determined with the substrates Bz-Arg-pNA, tos-Gly-Pro-Arg-pNA, and D-Phe-Pip-Arg-pNA at pH 7.9 and 37 °C. The value of k_{cat} did not vary with viscosity for any of the substrates. The value of k_{cat}/K_m for Bz-Arg-pNA was also found to be independent of the viscosity of the assay (data not shown). In contrast, the magnitude of

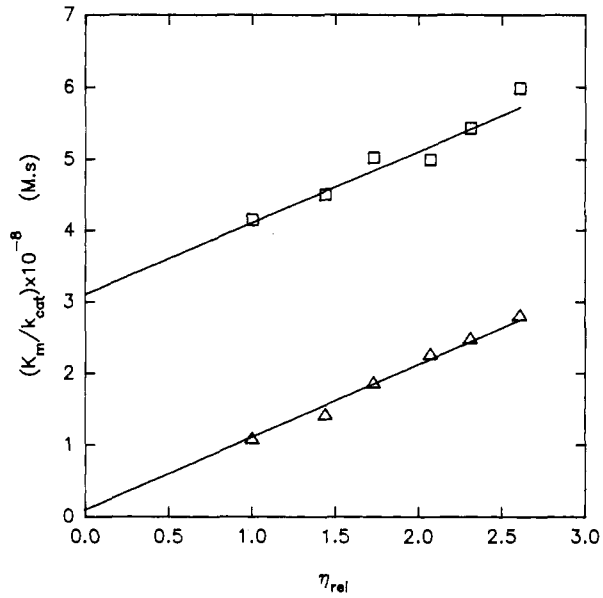


FIGURE 4: Effect of viscosity on k_{cat}/K_m for *p*-nitroanilide substrates. The data for D-Phe-Pip-Arg-pNA (△) and tos-Gly-Pro-Arg-pNA (□) are plotted according to eq 6 and the lines drawn represent the fit of the data to this equation.

Table II: Effect of Viscosity on the Reaction Catalyzed by Thrombin with the Substrates D-Phe-Pip-Arg-pNA and tos-Gly-Pro-Arg-pNA[a]

substrate	parameter	
	$10^{-7}k_1$ (M^{-1} s^{-1})	k_2/k_{-1}
tos-Gly-Pro-Arg-pNA	9.95 ± 1.20	0.32 ± 0.06
D-Phe-Pip-Arg-pNA	9.85 ± 0.84	10.6 ± 13.7

[a] The data illustrated in Figure 4 were analyzed according to eq 6 to yield the estimates of the parameters. The standard errors of the parameters derived from the analysis are also given.

Table III: Solvent Isotope Effects on Reactions Catalyzed by Thrombin[a]

substrate	parameter	
	$^{D}k_{cat}$	$^{D}(k_{cat}/K_m)$
Bz-Arg-pNA	3.1 ± 0.2	2.9 ± 0.3
tos-Gly-Pro-Arg-pNA	2.6 ± 0.2	2.0 ± 0.1
D-Phe-Pip-Arg-pNA	2.7 ± 0.1	1.0 ± 0.1
Ac-D-Phe-Pro-Arg-pNA	3.2 ± 0.4	2.4 ± 0.1

[a] Estimates for k_{cat} and k_{cat}/K_m were determined from data obtained in H_2O or D_2O buffers as described under Experimental Procedures, and the ratios of the estimates are given together with the standard errors of the ratios.

k_{cat}/K_m for tos-Gly-Pro-Arg-pNA and for D-Phe-Pip-Arg-pNA decreased with increasing viscosity (Figure 4). The horizontal intercept for the plot of K_m/k_{cat} against relative viscosity (η_{rel}) with D-Phe-Pip-Arg-pNA approached zero, indicating that this substrate is very sticky (see Theory and Data Analysis). In contrast, the data for tos-Gly-Pro-Arg-pNA indicate a limited stickiness for this substrate. Analysis of the data of Figure 4 according to eq 6 yielded estimates of k_1 and k_2/k_{-1} for both substrates (Table II). Values of 0.32 ± 0.06 and 10.6 ± 13.7 were determined for the ratio k_2/k_{-1} with tos-Gly-Pro-Arg-pNA and D-Phe-Pip-Arg-pNA, respectively. Estimates of about 10^8 M^{-1} s^{-1} were obtained for k_1 with both substrates (Table II).

Solvent Isotope Effects. The D_2O solvent isotope effects on k_{cat} and k_{cat}/K_m were determined at pH 7.9 and 37 °C for each of the substrates (Table III). At this pH, each of the substrates showed a similar solvent isotope effect of 2.6–3.2

on k_{cat} ($^{D}k_{cat}$, Table III). In contrast, the solvent isotope effect on k_{cat}/K_m varied from 2.9 with Bz-Arg-pNA to 1.0 with D-Phe-Pip-Arg-pNA; an intermediate effect was observed with tos-Gly-Pro-Arg-pNA and Ac-D-Phe-Pip-Arg-pNA (Table III).

Discussion

Kinetic Mechanisms for the Cleavage of p-Nitroanilide Substrates. The general mechanism for the cleavage of *p*-nitroanilide substrates by thrombin is given in Scheme I. A variety of techniques has been used to determine the magnitude of the rate constants for the cleavage of four *p*-nitroanilide substrates. In order to assess the results of pH studies, it is necessary to know whether catalysis is rate limiting and whether the magnitude of k_{-1} is greater than that of k_2. Viscosity and solvent isotope effects have been used to assess the relative magnitude of k_{-1} and k_2 in the region of the pH optimum (pH 7.9). The effect of viscosity on an enzyme-catalyzed reaction could be due either to a general effect of viscosity on the structure of the enzyme or to a specific effect on diffusion-controlled steps. The fact that the k_{cat}/K_m value for Bz-Arg-pNA was independent of viscosity provides an assurance that the effects observed with other substrates were not due to a general perturbation of the enzyme's structure (Brouwer & Kirsch, 1982). The lack of an effect of viscosity on k_{cat}/K_m for Bz-Arg-pNA indicates that it is a nonsticky substrate (Cleland, 1986). Analysis of the effects of viscosity on k_{cat}/K_m for D-Phe-Pip-Arg-pNA and tos-Gly-Pro-Arg-pNA provided estimates for k_1 and the ratio k_2/k_{-1} (Table II). While both substrates associated with the enzyme at about the same rate ($k_1 \approx 10^8$ M^{-1} s^{-1}), D-Phe-Pip-Arg-pNA was very sticky ($k_2 \gg k_{-1}$) and tos-Gly-Pro-Arg-pNA displayed limited stickiness ($k_2 \approx k_{-1}$). Solvent isotope effects on k_{cat}/K_m also provide an indication of the stickiness of the substrate. The value of 1.0 for $^{D}(k_{cat}/K_m)$ with D-Phe-Pip-Arg-pNA allows this substrate to be classified as sticky also on the basis of solvent isotope effects. For Bz-Arg-pNA, $^{D}(k_{cat}/K_m)$ was about equal to $^{D}k_{cat}$, which is expected for a nonsticky substrate when acylation is rate limiting (see Theory and Data Analysis). The other two substrates (tos-Gly-Pro-Arg-pNA and Ac-D-Phe-Pip-Arg-pNA) exhibited values of $^{D}(k_{cat}/K_m)$ that were significantly greater than 1.0 but less than that observed for Bz-Arg-pNA, which suggests that the degree of stickiness of these substrates is limited. For tos-Gly-Pro-Arg-pNA, the suggestion is consistent with the observed viscosity effects (Table II). The large solvent isotope effects seen on k_{cat} (Table III) indicate that catalysis rather than product release was rate limiting for all substrates (Cleland, 1977, 1986).

pH Dependence of k_{cat}/K_m. The most useful pH profiles for determining the pK_a values of groups involved in binding and catalysis are those for a poor substrate and for a competitive inhibitor. Although pK_a values determined from a k_{cat}/K_m profile for a good substrate may be displaced due to the stickiness of the substrate, the analysis of the pH dependence of k_{cat}/K_m for a poor substrate and K_i for a competitive inhibitor will always yield the correct pK_a values (Cleland, 1977, 1986). On the basis of the observed viscosity and D_2O solvent isotope effects (Tables II and III), Bz-Arg-pNA can be classified as a nonsticky substrate. Thus, the pK_a values of 7.57 and 9.09 observed in the k_{cat}/K_m profile for Bz-Arg-pNA should represent the true pK_a values of the groups involved in binding and catalysis (Table I). Analysis of the pH dependence of the K_i value for the competitive inhibitor DAMPA yielded similar estimates of 7.56 and 8.96 for the pK_a values, and this result confirms the correctness of the pK_a values observed in the k_{cat}/K_m profile for Bz-Arg-pNA. The pK_a values determined from the k_{cat}/K_m profiles for tos-Gly-Pro-Arg-pNA and Ac-D-Phe-Pip-Arg-pNA were not significantly displaced from those observed with Bz-Arg-pNA (Table I). These observations indicate that these two substrates have limited stickiness in accordance with the viscosity and solvent isotope effects seen with these substrates (Tables II and III). pK_a values of about 7.0 and 9.0 have previously been observed in the k_{cat}/K_m profiles for other serine proteases and have been attributed to the active-site histidine (His57) and the α-amino group of Ile16 (Fersht, 1985). It seems probable that these groups are also responsible for the pK_a values observed with thrombin. The temperature dependence seen for the pK_a values is consistent with this assignment. From the temperature dependence of pK_1 for tos-Gly-Pro-Arg-pNA, an estimate for ΔH_{ion} of 22 ± 8 kJ mol^{-1} was determined; this value is within the range expected for an imidazole group. The estimated ΔH_{ion} for the other group was 43 ± 7 kJ mol^{-1}, which is consistent with the ionization of a primary amino group being responsible for pK_2 (Tipton & Dixon, 1979).

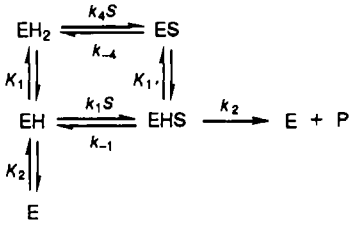
In comparison to the pK_a values for the other three substrates, those observed for the k_{cat}/K_m profile of D-Phe-Pip-Arg-pNA were displaced. The values observed for pK_1 and pK_2 were respectively 0.9 and 0.5 unit lower than those observed with Bz-Arg-pNA (Table I). The lower pK_a value for pK_1 is consistent with the stickiness of D-Phe-Pip-Arg-pNA (Tables II and III). The observed pK_a for a sticky substrate is related to the true value by (Cleland, 1977, 1986)

$$pK_1(obs) = pK_1 - \log (1 + k_2/k_{-1}) \qquad (14)$$

Thus, the difference between the pK_1 values for Bz-Arg-pNA and D-Phe-Pip-Arg-pNA can be used to calculate an estimate of 6.2 ± 0.9 for the ratio k_2/k_1. This estimate for k_2/k_{-1} is within the same range as that obtained from the viscosity effects (Table II). The accuracy of the estimate from the pH studies is, however, considerably better. The value of pK_2 determined from the k_{cat}/K_m profile for D-Phe-Pip-Arg-pNA is expected to show a simlar displacement to that seen for pK_1 but the observed pK_a should in this case be higher than the one seen with Bz-Arg-pNA (Cleland, 1977, 1986). The predicted value for pK_2(obs) would be 9.95 and this pK_a value might not be observed with the pH range used. The estimate for pK_2 obtained for D-Phe-Pip-Arg-pNA was actually lower than the one found with Bz-Arg-pNA (Table I). Examination of the crystal structure of the D-Phe-Pro-ArgCH_2–thrombin complex indicates that the α-amino group of the D-phenylalanine of the inhibitor makes a hydrogen bond to the carbonyl of Gly216 and possibly also weak salt bridges with the carboxylates of Glu192 and Glu217 (Bode et al., 1989). Similar interactions should also be possible with the α-amino group of D-Phe-Pip-Arg-pNA. Hydrogen bonds in which one of the partners is charged are known to be stronger than those in which both partners are uncharged (Fersht, 1987), and thus, it seemed possible that the pK_a observed in the k_{cat}/K_m profile could be due to a preferential binding of D-Phe-Pip-Arg-pNA molecules with protonated α-amino groups. The observed value for this pK_a of 8.6 is consistent with an α-amino group being responsible. In order to test this hypothesis, Ac-D-Phe-Pip-Arg-pNA was synthesized and its pH kinetics were determined. After acetylation of the α-amino group of D-Phe-Pip-Arg-pNA, the pK_a of 8.6 was no longer observed (Table I). Moreover, the determined pK_a values coincided with those for Bz-Arg-pNA because Ac-D-Phe-Pip-Arg-pNA was no longer a sticky substrate (Tables I and II). Interactions with the protonated α-amino group of D-Phe-Pip-Arg-pNA would appear to contribute to stickiness of this substrate.

An approximate estimate can be obtained for the contri-

Scheme II



bution of the charged α-amino group to catalysis by applying the relationship (Fersht, 1985)

$$\Delta\Delta G = -RT \ln [(k_{cat}/K_m)_0/(k_{cat}/K_m)_{Ac}] \quad (15)$$

where $\Delta\Delta G$ is the contribution of the α-amino group to transition-state stabilization and $(k_{cat}/K_m)_0$ and $(k_{cat}/K_m)_{Ac}$ are the pH-independent values of k_{cat}/K_m for D-Phe-Pip-Arg-pNA and Ac-D-Phe-Pip-Arg-pNA, respectively. By using eq 15 together with the values given in Table I, an estimate of 9.3 kJ mol^{-1} can be calculated for $\Delta\Delta G$. It should be noted that the calculated value of $\Delta\Delta G$ could under- or overestimate the contribution of the charged α-amino group depending on whether the acetyl group in Ac-D-Phe-Pip-Arg-pNA is involved in favorable or unfavorable interactions. The value calculated does not, however, seem an unreasonable estimate for the contribution of a hydrogen bond in which one of the partners is charged (Fersht, 1987).

pH Dependence of the Binding of the Substrate Analogue DAMPA. The dependence of the K_i value for DAMPA indicated that groups with pK_a values of 7.56 and 8.98 had to be respectively unprotonated and protonated for optimal binding of the inhibitor. These pK_a values correspond well with those observed in the pH profile of k_{cat}/K_m for Bz-Arg-pNA (Table I), and thus, the pK_a values can be assigned with some certainty to His57 and the α-amino group of Ile16. DAMPA has no groups that could be expected to ionize with the observed pK_a values. The observation that the α-amino group of Ile16 must be protonated for optimal binding of DAMPA is consistent with other studies that indicate that the protonated form of this residue is essential for the active conformation of serine proteases (Fersht, 1985). The requirement for His57 to be unprotonated for DAMPA binding is consistent with the structures modeled by Bode et al. (1990) of complexes between thrombin and the DAMPA-like inhibitors NAPAP [N^{α}-[(2-naphthylsulfonyl)glycyl]-DL-(*p*-amidinophenylalanyl)-piperidine) and MQPA [(2*R*,4*S*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid]. In these structures, the piperidine rings of the inhibitors are packed tightly against His57. If DAMPA binds to thrombin in a similar way, the tight packing of its piperidine ring against His57 would lead to an uncompensated positive charge if the histidyl residue were protonated. Thus, the binding of DAMPA to the unprotonated form of His57 would be favored.

pH Dependence of k_{cat}. The pH profile of k_{cat} for Bz-Arg-pNA displayed a half-bell-shaped curve with a pK_a of 6.3. Such curves have been previously observed for serine proteases (Fersht, 1985). This pH dependence can be interpreted in terms of the mechanism presented in Scheme II. Bz-Arg-pNA (S) can bind to the EH and EH_2 forms of the enzyme but cannot bind to the E form. This mechanism predicts that the pK_a for the conversion of EH to E (the ionization of the α-amino group of Ile16) will be seen in the k_{cat}/K_m profile but will not be seen in the k_{cat} profile (Cleland, 1977, 1986); this was in fact observed. The pK_a determined from the pH dependence of k_{cat} corresponds to that of the active-site histidine in the Michaelis complex, whereas the value determined from the k_{cat}/K_m profile corresponds to its pK_a in the free enzyme. Comparison of the values for this group seen in the two profiles indicates that the binding of Bz-Arg-pNA has resulted in a decrease in the pK_a of His57 by 1.2 units.

For the other substrates, the pH dependence of k_{cat} was more complicated and did not appear to reflect the ionization of any group that was essential for catalysis. Thus, the pK_a of the active-site histidine seems to be displaced by the binding of these substrates to a value outside of the pH range used. With the substrate tos-Gly-Pro-Arg-pNA, the value of k_{cat} decreased from pH 9 to 6, but the plot of log k_{cat} versus pH did not approach a slope of 1 even at the lowest pH values tested (Figure 2). The value of k_{cat} for D-Phe-Pip-Arg-pNA and Ac-D-Phe-Pip-Arg-pNA also varied little with pH; pH optima between 7.5 and 8.5 were observed with the value of k_{cat} decreasing to new plateau values above and below this range (Figure 2). A similar pH dependence for k_{cat} with D-Phe-Pip-Arg-pNA has previously been reported (De Cristofaro & Di Cera, 1990). A possible explanation for the observed variation of k_{cat} with pH for these three substrates is that, within the pH range tested, catalysis is affected by the ionization of one or more groups that are not directly involved in catalysis but whose ionization states influence the rate of catalysis to a limited extent. In this case, when the nonessential groups ionize, the value of k_{cat} changes from one plateau value to another. Alternatively, plots similar to those observed in Figure 2 can occur when the substrate is sticky and protons dissociate from the Michaelis complex more slowly than this complex undergoes catalysis, i.e., when both substrate and protons are sticky (Cleland, 1977, 1986). This kinetic explanation for the observed shape of the k_{cat} profiles could apply to the sticky substrate D-Phe-Pip-Arg-pNA but it can be rejected for the other two substrates, which are not sticky.

ACKNOWLEDGMENTS

We thank Drs. W. Bode and J. F. Morrison for helpful discussions and Mr. P. Müller for technical assistance.

Registry No. His, 71-00-1; Ile, 73-32-5; thrombin, 9002-04-4; benzoyl-Arg-pNA, 6208-93-1; D-Phe-pipecolyl-Arg-pNA, 64815-81-2; tosyl-Gly-Pro-Arg-pNA, 65316-83-8; acetyl-D-Phe-pipecolyl-Arg-pNA, 135944-79-5; *N-t*-Boc-di-Cbz-L-arginine, 51219-19-3; 4-methylpiperidine, 626-58-4; dansyl chloride, 605-65-2; N^{α}-dansyl-L-arginine-4-methylpiperidine amide, 55381-18-5.

REFERENCES

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467–3475.

Bode, W., Turk, D., & Stürzebecher, J. (1990) *Eur. J. Biochem. 193*, 175–182.

Brouwer, A. C., & Kirsch, J. F. (1982) *Biochemistry 21*, 1302–1307.

Chang, J.-Y. (1986) *Biochem. J. 240*, 797–802.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 237–387.

Cleland, W. W. (1986) in *Investigations of Rates and Mechanisms of Reactions* (Bernasconi, C. F., Ed.) Vol. 6, pp 791–870, Wiley, New York.

Cornish-Bowden, A., & Endrenyi, L. (1981) *Biochem. J. 193*, 1005–1008.

De Cristofaro, R., & Di Cera, E. (1990) *J. Mol. Biol. 216*, 977–985.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Fenton, J. W., II (1981) *Ann. N.Y. Acad. Sci. 370*, 468–495.

Fenton, J. W., II, & Bing, D. H. (1986) *Semin. Thromb. Hemostasis 12*, 200-208.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, Freeman, New York.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301-304.
Fromm, H. J. (1975) *Initial Rate Enzyme Kinetics*, Springer, Berlin.
Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J. 9*, 2361-2365.
Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 91-117.
Lottenberg, R., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 558-564.
Lottenberg, R., Christensen, U., Jackson, C. M., & Coleman, P. L. (1981) *Methods Enzymol. 80*, 341-361.
Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539-557.
Polgár, L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) pp 159-200, Elsevier, Amsterdam.
Rydel, T. J., Ravichrandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 245*, 277-280.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Tipton, K. F., & Dixon, H. B. F. (1979) *Methods Enzymol. 63*, 183-234.

Role of Interactions Involving C-Terminal Nonpolar Residues of Hirudin in the Formation of the Thrombin-Hirudin Complex

Andreas Betz, Jan Hofsteenge, and Stuart R. Stone*,‡
Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland
Received May 6, 1991; Revised Manuscript Received July 29, 1991

ABSTRACT: The role of interactions involving C-terminal nonpolar residues of hirudin in the formation of the thrombin-hirudin complex has been investigated by site-directed mutagenesis. The residues Phe56, Pro60, and Tyr63 of hirudin were replaced by a number of different amino acids, and the kinetics of the inhibition of thrombin by the mutant proteins were determined. Phe56 could be replaced by aromatic amino acids without significant loss in binding energy. While substitution of Phe56 by alanine decreased the binding energy (ΔG_b° by only 1.9 kJ mol^{-1}, replacement of this residue by amino acids with branched side chains caused larger decreases in ΔG_b°. For example, the mutant Phe56→Val displayed a decrease in ΔG_b° of 10.5 kJ mol^{-1}. Substitution of Pro60 by alanine or glycine resulted in a decrease in ΔG_b° of about 6 kJ mol^{-1}. Tyr63 could be replaced by phenylalanine without any loss in binding energy, and replacement of this residue by alanine caused a decrease of 2.2 kJ mol^{-1} in ΔG_b°. Substitution of Tyr63 by residues with branched side chains resulted in smaller decreases in ΔG_b° than those seen with the corresponding substitutions of Phe56; for example, the mutant Tyr63→Val showed a decrease in binding energy of 5.1 kJ mol^{-1}. The effects of the mutations are discussed in terms of the crystal structure of the thrombin-hirudin complex.

Hirudin is a specific tight-binding inhibitor of thrombin. Its tertiary structure in solution as determined by two-dimensional NMR spectroscopy indicates that it is composed of a relatively compact N-terminal core domain (residues 3-49) and a disordered C-terminal tail comprising residues 50-65 (Clore et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989). In the crystal structure of the complex, the C-terminal tail of hirudin is bound in a long groove on the surface of thrombin that is flanked by positively charged groups (Rydel et al., 1990; Grütter et al., 1990) and has been termed the anion-binding exosite (Fenton, 1989). Electrostatic interactions between the anion-binding exosite and acidic residues in the C-terminal region of hirudin appear to be important for the formation of the thrombin-hirudin complex (Braun et al., 1988; Stone et al., 1989; Betz et al., 1991). In addition, 5 of the 11 C-terminal residues of hirudin are involved in nonpolar interactions with this exosite (Rydel et al., 1990). Phe56′[1] is buried in a hydrophobic pocket as shown in Figure 1 and makes 14 intermolecular contacts less than 4 Å (Rydel et al., 1990). Moreover, the aromatic rings of Phe56′ and Phe34 of thrombin are perpendicular to each other, and this arrangement is considered to be optimal for a favorable interaction between aromatic rings (Burley & Petsko, 1985). Synthetic peptides based on the sequence of the C-terminal tail have been shown to inhibit the cleavage of fibrinogen by thrombin (Krstenansky et al., 1990), and the aromatic nature of Phe56′ appears to be important for the binding of these peptides to thrombin (Krstenansky et al., 1987). After Phe56′, the most important nonpolar residues in the C-terminal region of hirudin appear to be Pro60′ and Tyr63′ (see Figure 1). Both these residues make numerous nonpolar contacts with thrombin; Pro60′ makes 7 intermolecular contacts less than 4 Å while Tyr63′ makes 10 such contacts (Rydel et al., 1990).

Although the crystallographic studies suggest that nonpolar interactions with the C-terminal region of hirudin are important in the stabilization of the complex, quantitative data on the strength of these interactions for individual residues

* Address correspondence to this author.
‡ Present address: Department of Haematology, University of Cambridge, MRC Centre, Hills Rd., Cambridge CB2 2QH, United Kingdom.

[1] Residues in rhir are distinguished from those in thrombin by the use of primed numbers; e.g., Phe56′ represents the phenylalanine at position 56 in rhir. The numbering of residues in thrombin is that of Bode et al. (1989) which is based on chymotrypsin numbering.

0006-2960/91/0430-9848$02.50/0