Linkage between Proton Binding and Amidase Activity in Human α-Thrombin: Effect of Ions and Temperature†

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ABSTRACT: The amidase activity of human α-thrombin has been studied at steady state in the pH range 6–10, as a function of NaCl concentration from 1 mM to 1 M and temperature from 10 to 40 °C. The Michaelis–Menten constant, K_m, shows a bell-shaped dependence over this pH range with a minimum around pH 7.5 in the presence of 0.1 M NaCl at 25 °C. The catalytic constant, k_{cat}, also has a bell-shaped pH dependence with multiple inflection points that are more evident at low NaCl concentrations and a maximum around pH 8.2 in the presence of 0.1 M NaCl at 25 °C. A detailed analysis of the results in terms of a general linkage scheme has allowed a thorough characterization of the linkage between proton and substrate binding and its dependence on NaCl concentration, as well as the relevant entropic and enthalpic contributions to binding and catalytic events. Formulation of detailed partition functions for each enzyme intermediate involved in the catalytic cycle suggests that (at least) three groups are responsible for the control of thrombin amidase activity as a function of pH. One group is to be identified with the active site His, due to its pK values in the free enzyme and the adduct and its enthalpy of ionization. The effect of NaCl concentration on amidase activity seems to be extremely specific. Comparative steady-state measurements carried out in the presence of NaCl, NaBr, NaI, KCl, and $MgCl_2$ show that human α-thrombin is capable of discriminating among different cations and anions. This suggests that small ions participate as allosteric effectors in the regulation of thrombin activity. The linkage with NaCl is strongly pH dependent and increases with decreasing pH. The present results provide information on the basic aspects of human α-thrombin activity and regulation and enable a rigorous thermodynamic approach to other important regulatory interactions in human α-thrombin and its structurally perturbed derivatives.

Thrombin is a serine protease characterized by an extreme selectivity in cleaving fibrinogen to form fibrin (Higgins et al., 1983; Lewis et al., 1987; Vali & Scheraga, 1988) and a remarkable flexibility of interaction with a number of effector molecules that modulate its enzymatic activity (Conery & Berliner, 1983; Berliner, 1984; Fenton, 1986; Stone & Hofsteenge, 1986). The nature of thrombin specificity has recently been addressed in structural terms by detailed X-ray analysis (Bode et al., 1989; Rydel et al., 1990; Chow et al., 1990), and important aspects have emerged on the structural bases of thrombin function. The increasing availability of high-resolution structural details strongly motivates a systematic experimental approach to the functional properties of the enzyme and makes the system a suitable candidate for quantitative biophysical studies. The aim of these studies is to elucidate the physicochemical mechanisms of regulation of thrombin activity, along with the basic driving forces responsible for the structural changes of functional significance and their thermodynamic aspects. The strategy to be followed includes a combination of theoretical, computational, and experimental methods. Of particular importance has been the recent development of a thermodynamic scheme for the linkage between thrombin catalytic activity and the binding of effector molecules (De Cristofaro & Di Cera, 1990). This treatment is an extension of the equilibrium theory of ligand binding and linkage (Wyman, 1964; Di Cera et al., 1988; Di Cera, 1990; Wyman & Gill, 1990) to macromolecular systems working at steady state and provides a rigorous thermodynamic framework for the description of regulatory interactions in the thrombin system. The general approach makes it possible to globally analyze experimental data obtained at steady state over a wide range of solution conditions and to resolve the relevant thermodynamic parameters involved in the linkage scheme with high precision. We have recently applied this approach to the study of the effects of protons on human α-thrombin activity using a synthetic amide substrate (De Cristofaro & Di Cera, 1990). We have shown that although substrate binding to the catalytic pocket seems to be controlled by only two ionizable groups, as in the case of other serine proteases (Fersht & Renard, 1974; Fersht, 1985), nevertheless the catalytic activity of the enzyme is more complex and appears to be controlled by several proton-linked ionizable groups. These findings have brought out the idea that the unique structural features whereby thrombin derives its extreme specificity might also be responsible for peculiar regulatory interactions. Indeed, thrombin is characterized by an unusual richness of charged residues, twice the content of trypsin or chymotrypsin (Bode et al., 1989), and this feature agrees well with the experimental observation of a regulation of the catalytic activity by protons that differs significantly from that of other serine proteases.

In this paper we further our investigation of the basic regulatory aspects of thrombin activity by exploring the pH dependence of binding and catalytic events over a wide range

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Table I: Effect of Temperature on the pH of the Triple Buffer BisTris/Tris/CHES in the Presence of 0.1 M NaCl and 0.1% PEG 8000[a]

pH	$\Delta pH/\Delta T$	σ
6.00	−0.0144 ± 0.0009 (−0.0171 ± 0.0013 BisTris)	0.053
6.50	−0.0156 ± 0.0008 (−0.0172 ± 0.0019 BisTris)	0.047
7.00	−0.0171 ± 0.0004 (−0.0161 ± 0.0016 BisTris)	0.028
7.50	−0.0211 ± 0.0007 (−0.0276 ± 0.0017 Tris)	0.044
8.00	−0.0235 ± 0.0008 (−0.0267 ± 0.0012 Tris)	0.046
8.50	−0.0244 ± 0.0007 (−0.0270 ± 0.0008 Tris)	0.040
9.00	−0.0224 ± 0.0006 (−0.0219 ± 0.0014 CHES)	0.036
9.50	−0.0224 ± 0.0007 (−0.0214 ± 0.0016 CHES)	0.044
10.00	−0.0213 ± 0.0009 (−0.0215 ± 0.0010 CHES)	0.054

[a] The coefficients $\Delta pH/\Delta T$ are the results of global analysis of four different sets of determinations. The values of $\Delta pH/\Delta T$ for each buffer component separately, under identical solution conditions, are given in parentheses. σ is the standard deviation of the fit in pH units.

of salt concentrations and temperatures. Here we apply our theoretical, computational, and experimental approaches to fully characterize the energetics of the relevant reactions involved in the linkage between thrombin activity and proton binding in terms of entropic and enthalpic components and quantify the contributions arising from binding of small ions. The results obtained in this study provide quantitative information on the functional energetics of the enzyme that can be used to structurally locate the specific residues involved in the control of binding and catalysis. The approach taken here also sets the stage of application of the same strategy to the study of structurally perturbed thrombins, such as the γ and ζ derivatives (Brezniak et al., 1990), which is likely to provide quantitative information on the linkage between functional properties and structural perturbations for this physiologically important enzyme.

Theory

In this section we summarize the important thermodynamic aspects that are relevant to the analysis of the linkage effects reported in this study. Although some of these aspects are discussed in detail elsewhere (De Cristofaro & Di Cera, 1990), they are given here in simplified form to make the present study self-contained.

The General Linkage Scheme. We start from the basic kinetic scheme for serine proteases (Fersht, 1985)

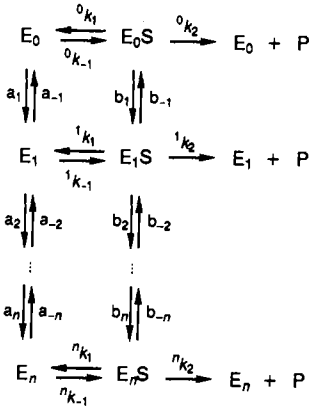
$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P' \qquad (1)$$

where E is the free enzyme and ES and EP denote the enzyme–substrate and enzyme–product adducts, while k_1, k_{-1}, k_2, and k_3 are the kinetic constants for substrate binding and dissociation (k_1 and k_{-1}), acylation (k_2), and deacylation (k_3). All rate constants in eq 1 are first-order except k_1 that is pseudo-first-order and is equal to $k_1 = k_1^*[S]$, where [S] is the substrate concentration. When serine proteases react with amide substrates, which is the case dealt with in this study, acylation is rate limiting and substrate dissociation is fast as compared to catalysis over a wide pH range (Bender et al., 1964; Curragh & Elmore, 1964; Zerner & Bender, 1964; Brandt et al., 1967; Fastrez & Fersht, 1973; Fresht, 1985). This leads to a classical Michaelis–Menten kinetics where the EP intermediate does not accumulate and can be dropped from the kinetic scheme. The catalytic activity at steady state is given by

$$v = e_T \frac{k_{cat}[S]}{K_m + [S]} \qquad (2)$$

where $e_T \equiv [E] + [ES] + [EP]$ is the total enzyme concentration, while $k_{cat} = k_2$ and $K_m = k_{-1}/k_1^* \equiv K_d$ are the relevant kinetic parameters.

The effect of protons (or any other linked ligand) on the kinetics can be addressed by a suitable extension of the reaction scheme in eq 1, with the manifold of EP species being dropped, as follows (De Cristofaro & Di Cera, 1990)



(3)

where n is the total number of linked protons and the a's and b's refer to proton binding and dissociation in the free enzyme and the adduct, respectively. All rate constants are first-order except the a_j's and b_j's (j = 1, 2, ..., n) that are pseudo-first-order and equal to $a_j = a_j^* h$ and $b_j = b_j^* h$, with h being the proton activity. Also ${}^j k_1 = {}^j k_1^*[S]$, as already noted. The catalytic constant ${}^j k_2$ is the acylation rate of the j-protonated enzyme form. The reaction scheme in eq 3 is perfectly general and makes no assumption on the values of the rate constants or the number of sites for the effector molecule. The steady-state velocity of product formation, v, for eq 3 in the general case can be obtained by application of the celebrated King–Altman algorithm (King & Altman, 1956) or else by the elegant diagram method (Hill, 1977). The exact analytical form of the solution can be written as the ratio of two polynomial expansions in h and [S], with the coefficients being cumbersome relationships of the rate constants involved in the scheme. The degree of the polynomials can be as high as $n + 1$ in [S] and $2n$ in h, depending upon the values of the rate constants, and the complexity of v is such that the resolvability of the rate constants from analysis of experimental data is drastically hindered, even in the simplest case of $n = 1$ (Botts & Morales, 1954). There is, however, very little need to invoke a general solution for eq 3 for practical purposes, and especially when dealing with small linked ligands such as protons. In fact, in this case effector binding and dissociation reactions are fast compared to catalytic rates (Fersht, 1985), and all intermediates in the E and ES manifolds are in a regime of *quasi-equilibrium* (Hill, 1980). Also, a Michaelis–Menten kinetics implies that the steady-state solution for v must contain polynomial expressions of first degree in [S]. The quasi-equilibrium regime leads to a detailed balancing solution of the relevant kinetic equations (De Cristofaro & Di Cera, 1990), and the kinetic mass action law yields v as the ratio of two polynomials that are first degree in [S] and nth degree in h. Under these conditions, the mathematical treatment of the reaction scheme in eq 3 can be cast solely in terms of the ensemble properties $\{E\} = [E_0] + [E_1] + ... + [E_n]$ and $\{ES\} = [E_0S] + [E_1S] + ... + [E_nS]$, with the relevant kinetic constants being average values over each manifold (De Cristofaro & Di Cera, 1990), i.e.,

$$\{E\} \underset{\langle k_{-1}\rangle}{\overset{\langle k_1\rangle}{\rightleftharpoons}} \{ES\} \xrightarrow{\langle k_2\rangle} \{E\} + P \qquad (4)$$

where $\langle k_1\rangle = \langle k_1^*\rangle[S]$. The steady state expression for v is

given again by the classical Michaelis–Menten eq 2 with

$$K_m = \langle k_{-1}\rangle/\langle k_1^*\rangle = {}^0k_{-1}\sum_{j=0}^{n}A_jh^j/{}^0k_1^*\sum_{j=0}^{n}B_jh^j = {}^0K_dZ_E/Z_{ES} \quad (5)$$

$$k_{cat} = \langle k_2\rangle = \sum_{j=0}^{n}{}^jk_2B_jh^j/\sum_{j=0}^{n}B_jh^j = \sum_{j=0}^{n}{}^jk_2B_jh^j/Z_{ES} \quad (6)$$

The parameter $A_j = a_1^*a_2^*...a_j^*/a_{-1}a_{-2}...a_{-j}$ is the overall association constant for the binding reaction $E_0 + jH^+ = E_j$ (H^+ denotes proton), and likewise $B_j = b_1^*b_2^*...b_j^*/b_{-1}b_{-2}...b_{-j}$ is the overall assocation constant for the reaction $E_0S + jH^+ = E_jS$. The functions Z_E and Z_{ES} are the canonical partition functions of the free enzyme and the adduct. These functions encapsulate the underlying linkage effects in the system and provide the connection between kinetic and thermodynamic properties of eq 3.

Equations 5 and 6 give the exact solution of eq 3 under the assumption of fast proton equilibration and are isomorphic to those obtained when dealing with linkage effects at equilibrium. The expression for K_m is identical with the classical pH dependence of a dissociation constant (Edsall & Wyman, 1958; Wyman, 1964), and therefore differentiation of ln K_m yields

$$\text{d ln } K_m/\text{d ln } h = H_E^+ - H_{ES}^+ = \Delta H^+ \quad (7)$$

where H_E^+ and H_{ES}^+ are the number of protons bound to E and ES, respectively. The pH dependence of ln K_m yields a measure of the total number of protons exchanged (released or uptaken) upon substrate binding to the enzyme. The value of ΔH^+ is a quantitative expression of the linkage between proton and substrate binding to the enzyme. The expression for k_{cat} is more elaborate since the terms at the numerator are distorted by the catalytic constants ${}^jk_2$'s. Both expressions, however, are simple enough for practical application. Of particular importance is the fact that the partition function Z_{ES} enters the definition of both k_{cat} and K_m, which introduces a constraint in the computational analysis and interpretation of experimental data and greatly improves the resolvability of the $3n + 2$ independent parameters involved in the definition of k_{cat} and K_m.

The proton binding equilibria can further be decoupled in terms of the contributions arising from n independent ionizable groups. This assumption is very often consistent with the experimental facts (see Results) and can be made without loss of generality. The relevant partition functions can be rewritten as

$$Z_E = (1 + \alpha_1h)(1 + \alpha_2h)...(1 + \alpha_nh) \quad (8)$$

$$Z_{ES} = (1 + \beta_1h)(1 + \beta_2h)...(1 + \beta_nh) \quad (9)$$

where α_j and β_j are the proton binding constants of the jth ionizable group of the E and ES forms, respectively. The pK value of each group is simply the logarithm of the proton binding constant.

Ion Effects. The ionization reactions involving the proton can be affected by various factors that provide useful information on the nature of the ionizable group or macromolecular structural changes that are energetically significant. Linkage effects studied in two or more "dimensions", each one reflecting the contribution of a particular ligand, provide deeper insights into the energetics of the system and greatly expand the information that can be exploited in the computational analysis of experimental data. Extension of the mathematical treatment to the case of multiple effectors is straightforward. When the quasi-equilibrium regime is extended to another effector other than the proton, then the proton binding constants A_j and B_j in eqs 5 and 6 become functions of the activity of the second effector, y, and so do the partition functions Z_E and Z_{ES}. The dependence of ln $A_i(y)$ on y gives the linkage relationship

$$\text{d ln } A_i(y)/\text{d ln } y = Y_{Ei} - Y_{E0} = \Delta Y_i \quad (10)$$

that is, the number of molecules of the second effector exchanged in going from E_0 to the i-protonated intermediate E_i or else the differential binding of Y to the enzyme forms E_0 and E_i. Similar arguments hold for the dependence of ln $B_i(y)$ on y. The dependence of α_i on y gives the number of Y molecules linked to protonation of the ith ionizable group in the free enzyme and likewise for β_i.

When the second effector is an ion, say chloride, the effects on the protonation reactions linked to substrate binding and catalysis must include the binding contributions of both the anion and the cation associated with it to form the salt. In addition to specific binding reactions, "ionic strength" effects should also be expected as a result of possible influences of the salt on the activity coefficients of water, proton, substrate, and the various forms of the enzyme (Tanford, 1962, 1969; Record et al., 1978). Specifically, in the presence of a salt, eq 10 should be rewritten as

$$\frac{\text{d ln } A_i(a_\pm)}{\text{d ln } a_\pm} = \Delta Y_+ + \Delta Y_- - \omega\Delta W + \frac{\text{d ln } \gamma_E^0/\gamma_{Ei}^0}{\text{d ln } a_\pm} + \frac{i\,\text{d ln } a_h}{\text{d ln } a_\pm} \quad (11)$$

where $a_\pm$ is the mean activity of the salt, ΔY_+ and ΔY_- denote the differential binding of the cation and the associated anion, ΔW is the differential hydration, ω is a constant proportional to the molal concentration of the salt, γ_E^0 and γ_{Ei}^0 are the standard activity coefficients of E and E_i, and a_h is the proton activity. The effect of salt on the equilibrium constant A_i not only includes differential binding of both the cation and the anion but also reflects differential hydration and aspecific ionic strength effects such as Debye–Hückel screening on the enzyme charges. Discrimination among these effects, and especially between specific linkage effects due to ion binding and aspecific ionic strength effects due to screening, is always very important and necessary to correctly establish the contribution of the various components to the effects observed experimentally.

Temperature Effects. Temperature effects play a key role in linkage thermodynamics (Di Cera et al., 1988; Wyman & Gill, 1990). Hence, one naturally expects that a great deal of information can be gained from the study of the relevant ionization reactions involving the protons at different temperatures. As indicated by free amino acids in aqueous solutions, the enthalpic contribution to the ionization reaction of carboxyl groups is negligible, while that of amino groups ranges from about 7 to 14 kcal/mol (Edsall & Wyman, 1958). The entropy of ionization is usually negative because ion formation produces intense local electrostatic fields that inhibit the freedom of motion of water dipoles. Ionization reactions in macromolecular systems can be perturbed by additional effects arising mostly from electrostatic interactions, as illustrated by the sensitivity of pK values to the structure of the local environment, and therefore they may not conform to the rules derived from the behavior of simple amino acids in solution (Edsall & Wyman, 1958; Tanford, 1962; Knowles, 1976). However, the information collected from studies of ionization reactions at different temperatures is absolutely necessary to establish either the possible nature of the residues involved in the control of binding and catalytic events and/or

the nature of the perturbation, if any, arising from interaction with the structural microenvironment surrounding the residues. Temperature effects also provide information on the enthalpic and entropic contributions to substrate binding at different pH, as well as on the thermodynamic parameters of catalytic events.

Under the assumption that proton binding equilibria can be cast in terms of n independent ionizable groups, the temperature dependence of α_i, reflecting the proton affinity of the ith ionizable group in the E form, is given by integration of the Gibbs–Helmholtz equation

$$\partial \ln \alpha_i / \partial(1/T) = \Delta H_i / R \quad (12)$$

where R is the gas constant (1.98717 cal/mol/K), T is the absolute temperature, and ΔH_i is the standard enthalpy change associated with ionization (proton dissociation) of the ith group. The integral of eq 12, under the assumption of a constant ΔH_i over the temperature range examined, is given by (Gill et al., 1985)

$$\alpha_i = \alpha_i^0 \exp[\Delta H_i(\tau - \tau_0)/R] \quad (13)$$

where α_i^0 is the value of α_i at the standard temperature T_0 = 298.15 K, while $\tau = 1/T$ and $\tau_0 = 1/T_0$. Analogous expressions can be written for β_i. The relevant expression for ${}^0K_d$ is

$${}^0K_d = {}^0K_d^0 \exp[\Delta H_s(\tau - \tau_0)/R] \quad (14)$$

where ΔH_s is the standard enthalpy of substrate binding to the catalytic pocket. The approach used for integration of the Gibbs–Helmholtz equation can be extended to the Arrhenius law. Integration of this law for the acylation rate of the i-protonated intermediate yields

$${}^ik_2 = {}^ik_2^0 \exp[-\Delta E_i(\tau - \tau_0)/R] \quad (15)$$

where ΔE_i is the activation energy. Substitution of eqs 13–15 in the expressions for k_{cat} and K_m yields the analytical expressions to be used in the analysis of experimental data obtained at different temperatures.

Materials and Methods

Thrombin Preparation. Human α-thrombin was prepared as described elsewhere (Fenton et al., 1977). Contaminant β- and γ-thrombins (17% and 25%, respectively) were eliminated by cation-exchange chromatography on Amberlite CG-50 or alternatively on Bio-Rex 70. When Amberlite CG-50 was used, a Pharmacia-LKB XK16 column (1.6 × 40 cm) was equilibrated at 4 °C in 0.15 M NaCl and 50 mM Tris,[1] pH 8.00. A mixture of β- and γ-thrombins eluted shortly after the void volume, while α-thrombin eluted after raising the NaCl concentration to 0.75 M. When Bio-Rex 70 was used, the same column was equilibrated at 4 °C in 0.15 M NaCl and 50 mM Tris, pH 8.00. At this ionic strength a small peak was eluted, while a larger peak containing a mixture of β- and γ-thrombin was eluted after raising the ionic strength with 0.30 M NaCl. SDS–PAGE on 5–25% gradient gels and silver staining of fractions pooled from these peaks revealed the presence of denatured thrombin, as well as a mixture of β- and γ-thrombin. Neither peak contained either pure β- or γ-thrombin, contrary to some recent claims (Elion et al., 1986). α-Thrombin was eluted by further raising the ionic strength with 0.75 M NaCl. Notwithstanding slight differences in the gradient to be applied in the two cases, both resins yielded identical results as to the purity of α-thrombin, that was 99% pure as judged by SDS–PAGE on 5–25% gradient gels run under both reducing and nonreducing conditions. Thrombin concentration was measured with an extinction coefficient E_{280} = 1.83 mL/(mg·cm) and a molecular weight of 36 500 (Fenton et al., 1977). The concentration determined spectrophotometrically matched the active site concentration determined by titration with p-nitrophenylguanidinobenzoate hydrochloride (Chase & Show, 1967). Thrombin solutions of 2 μM concentration were stored in 50-μL vials at −80 °C until use.

Steady-State Measurements. Steady-state measurements of human α-thrombin amidase activity were made with the synthetic chromogenic peptide S-2238 purchased from Kabi (Stockholm, Sweden). The concentration of S-2238 was carefully measured at 342 nm with a Cary 3 dual-beam spectrophotometer before and after each experimental determination. All assays were performed by following the release of p-nitroaniline resulting from the hydrolysis of S-2238 at 405 nm with a Cary 3 dual-beam spectrophotometer, equipped with a 6 × 6 cell holder. The cell holder is thermostated within ±0.05 °C by a built-in Peltier apparatus connected to a circulating water bath. The Cary 3 spectrophotometer is interfaced to and fully controlled by an IBM PS/2 computer that collects and process the data prior to transfer of the results for data analysis to a Compaq 486/25/320 computer. Assays were performed with disposable polystyrene cuvettes under the desired solution conditions. Thrombin concentration was 1 nM in all assays, and 0.1% PEG 8000 was used under all solution conditions to prevent adsorbance of the enzyme to the cuvette walls.

The pH dependence of thrombin amidase activity was studied in the pH range 6–10 with a triple buffer containing 25 mM BisTris (pK_a = 6.5 at 25 °C), 25 mM Tris (pK_a = 8.0 at 25 °C), and 25 mM CHES (pK_a = 9.5 at 25 °C). The triple buffer was chosen to minimize complications arising from differential interactions of each component with the enzyme, as well as drastic changes in the ionic strength of the medium. The composition of the triple buffer is such that its ionic strength changes only very little, from about 0.03 to about 0.045, over the pH range studied (Ellis & Morrison, 1982). Solutions were titrated at the desired pH by using an Orion Research EA 940 expandable ion analyzer equipped with an automatic temperature compensation probe.

Substrate concentrations typically ranged from 0.5 to about 50 μM. Seven points were collected in each determination by stepwise 2-fold dilutions of the highest substrate concentration. The concentration of released p-nitroaniline was quantified by means of an extinction coefficient derived from the empirical expression

$$E_{405} = 9783.6 + 596.5I \quad (16)$$

where I is the ionic strength of the solution. The expression above was derived from the best-fit of data reported elsewhere (Lottenberg & Jackson, 1983) and is accurate within ±40.9 units with one standard deviation confidence. Under standard experimental conditions of I = 0.1, such an error induces an insignificant deviation (<0.5%) on the calculation of k_{cat}. Even at zero ionic strength, the deviation is well below the experimental error of k_{cat} that in accurate measurements can be as low as 1%. Velocity measurements of substrate hydrolysis taken over an interval of 60 s (3 points/s) were used to calculate initial rates by linear interpolation with the KINETICS

[1] Abbreviations: BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; BzArgNHNp, N-benzoyl-Arg-p-nitroanilide; CHES, 2-(N-cyclohexylamino)ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol); S-2238, H-D-Phe-pipecolyl-Arg-p-nitroanilide.

software of the Cary 3 spectrophotometer. The kinetic parameters, K_m and k_{cat}, were obtained as described under Data Analysis.

Temperature and Ion Effect Studies. The catalytic activity of human α-thrombin was explored over the pH range 6–10 under different conditions. First, steady-state measurements were conducted at pH 8.00 with different salts to assess the contribution of ionic strength effects. Specific anionic differential interactions with the enzyme were tested by measuring thrombin amidase activity in the presence of NaCl, NaBr, NaF, and NaI in the concentration range from 1 mM to 1 M. Analogous measurements in the presence of NaCl, KCl, and $MgCl_2$ in the concentration range from 1 mM to 1 M were taken to assess specific cationic differential interactions. The effect of NaCl in the concentration range from 1 mM to 1 M was then tested over the entire pH range. At each pH, initial velocity measurements were collected in a 7 × 7 matrix, with seven different concentrations of substrate and seven different concentrations of NaCl for each substrate concentration. The concentrations of NaCl were obtained by 3-fold stepwise dilutions starting from 1 M.

Temperature effects were studied in the temperature range 10–40 °C, with the triple buffer in the presence of 0.1 M NaCl and 0.1% PEG 8000. Each solution was first precisely titrated at the desired pH at 25 °C and then used for measurements over the entire temperature range. The change in pH with temperature of each solution was carefully measured by separate experiments on the triple buffer, and the $\Delta pH/\Delta T$ coefficients for all pH values employed in this study are given in Table I. The coefficients were computed by global analysis of four independent determinations of the pH values over the temperature range 4–45 °C. Newly prepared solutions of triple buffer were used for each determination and two Tris-specific flat-surface Corning electrodes were alternatively used to minimize any complication arising from possible drifts in the electrode readings over the temperature range explored. Control measurements of the $\Delta pH/\Delta T$ coefficients for each buffer component separately were also carried out in duplicate and are given in Table I in parentheses. These coefficients agree very well with the $\Delta pK_a/\Delta T$ values reported in the literature (Ellis & Morrison, 1982; Stoll & Blanchard, 1990). Measurement of the $\Delta pH/\Delta T$ coefficients allowed the calculation the pH of each solution at any given temperature and the construction of a matrix of K_m and k_{cat} values at a different pH and temperature.

Data Analysis. Initial velocity determinations were first analyzed according to eq 2 to yield the Michaelis–Menten parameters k_{cat} and K_m. The data were analyzed by nonlinear least-squares in the form collected experimentally, i.e., in a plot of v versus [S], and weighted uniformly. Parameter estimation was accomplished by the Marquardt method (Bard, 1974), and confidence intervals on the parameters were computed by F-testing at the cutoff of one standard deviation (68%). Thorough analysis of residuals according to "point perturbation analysis" (Di Cera et al., 1989) revealed no significant departure from random behavior and completely justified the use of a uniform weighting scheme, contrary to the commonly accepted assumption that the experimental error of initial velocity measurements is not uniform and proportional to v. Analysis of residuals is always necessary to assess the corectness of weighting schemes. Incorrect weighting schemes can result from either incorrect assumptions on the distribution of experimental errors or more often from linearization of the data in the form of the popular Lineweaver–Burk and Eadie–Hofstee plots aimed at avoiding nonlinear regression. Incorrect weighting can severely bias best-fit parameter values, unless one deals with highly accurate (≤2% error) measurements, as recently determined by extensive Monte Carlo simulations (Di Cera, 1991).

The values of k_{cat} and K_m collected at different pH's as a function of NaCl concentration or temperature were globally analyzed with analytical expressions derived from the linkage scheme in eq 3. At each NaCl concentration, the values of k_{cat} and K_m collected as a function of pH were fitted according to eqs 5 and 6 by using the minimal phenomenological scheme consistent with the whole set of experimental data. The data were analyzed in terms of two, three, or four proton-linked ionizable residues, and the χ^2 was computed in all cases for statistical evaluation. No significant difference was found between fitting equations cast in terms of three or four ionizable groups, but a drastic difference, more than 95% significant, was found between two and three ionizable residues. The minimum number of residues ($n = 3$ in eq 3) consistent with the experimental data was then chosen for all analyses. The explicit forms of Z_E and Z_{ES} were as follows

$$Z_E = (1 + \alpha_1 h)(1 + \alpha_2 h)(1 + \alpha_3 h) \quad (17)$$

$$Z_{ES} = (1 + \beta_1 h)(1 + \beta_2 h)(1 + \beta_3 h) \quad (18)$$

Hence,

$$K_m = {}^0K_d \frac{(1 + \alpha_1 h)(1 + \alpha_2 h)(1 + \alpha_3 h)}{(1 + \beta_1 h)(1 + \beta_2 h)(1 + \beta_3 h)} \quad (19)$$

$$k_{cat} = [{}^0k_2 + {}^1k_2(\beta_1 + \beta_2 + \beta_3)h + {}^2k_2(\beta_1\beta_2 + \beta_1\beta_3 + \beta_2\beta_3)h^2 + {}^3k_2\beta_1\beta_2\beta_3 h^3]/[(1 + \beta_1 h)(1 + \beta_2 h)(1 + \beta_3 h)] \quad (20)$$

were the fitting functions used in nonlinear least-squares analyses. The pK associated with each ionizable group was obtained from the logarithm of α or β. All independent parameters in eqs 19 and 20 were resolved with good accuracy from analysis of experimental data. The analysis of the pH dependence of k_{cat} and K_m was conducted at each NaCl concentration separately, and collection of the results yielded the change of the relevant parameters in eqs 19 and 20 as a function of salt concentration. Since α_3 was never found to be significantly different from β_3, the two parameters were constrained to be the same in all analyses.

In the case of the temperature studies, the complete data set of k_{cat} and K_m values obtained at different pH and temperature was globally analyzed by nonlinear least-squares. As in the case discussed above, the data were first analyzed in terms of two, three, and four ionizable residues, and again $n = 3$ was the minimum number of residues consistent with the whole set of data. Interestingly, the statistical significance in rejecting the case $n = 2$ as compared to $n = 3$ was even higher (≥ 99.2%), probably due to the increased number of degrees of freedom in the fitting problem. Each parameter in eqs 19 and 20 was expressed as a function of temperature by using the van't Hoff and Arrhenius expressions in eqs 13–15, so that

$$K_m = {}^0K_d{}^0 \exp[\Delta H_s(\tau - \tau_0)/R] \frac{Z_E(T)}{Z_{ES}(T)} \quad (21)$$

$$k_{cat} = \frac{P_{ES}(T)}{Z_{ES}(T)} \quad (22)$$

where

$$Z_E(T) = [1 + \alpha_1^0 \exp[^E\Delta H_1(\tau - \tau_0)/R]h][1 + \alpha_2^0 \times \exp[^E\Delta H_2(\tau - \tau_0)/R]h][1 + \alpha_3^0 \exp[^E\Delta H_3(\tau - \tau_0)/R]h] \quad (23)$$

$$Z_{ES}(T) = [1 + \beta_1^0 \exp[^{ES}\Delta H_1(\tau - \tau_0)/R]h][1 + \beta_2^0 \times \exp[^{ES}\Delta H_2(\tau - \tau_0)/R]h][1 + \beta_3^0 \exp[^{ES}\Delta H_3(\tau - \tau_0)/R]h] \quad (24)$$

$$P_{ES}(T) = {}^0k_2^0 \exp[-\Delta E_0(\tau - \tau_0)/R] + {}^1k_2^0 \exp[-\Delta E_1(\tau - \tau_0)/R]\{\beta_1^0 \exp[^{ES}\Delta H_1(\tau - \tau_0)/R] + \beta_2^0 \exp[^{ES}\Delta H_2(\tau - \tau_0)/R] + \beta_3^0 \exp[^{ES}\Delta H_3(\tau - \tau_0)/R]\}h + {}^2k_2^0 \times \exp[-\Delta E_2(\tau - \tau_0)/R]\{\beta_1^0\beta_2^0 \exp[(^{ES}\Delta h_1 + {}^{ES}\Delta H_2) \times (\tau - \tau_0)/R] + \beta_1^0\beta_3^0 \exp[(^{ES}\Delta H_1 + {}^{ES}\Delta H_3)(\tau - \tau_0)/R] + \beta_2^0\beta_3^0 \times \exp[(^{ES}\Delta H_2 + {}^{ES}\Delta H_3)(\tau - \tau_0)/R]h^2 + {}^3k_2^0\beta_1^0\beta_2^0\beta_3^0 \times \exp[(-\Delta E_3 + {}^{ES}\Delta H_1 + {}^{ES}\Delta H_2 + {}^{ES}\Delta H_3)(\tau - \tau_0)/R]h^3 \quad (25)$$

A total of 22 parameters involved in eqs 21–25 were resolved with good accuracy from global analysis of experimental data.

The robustness of the best-fit solution was thoroughly checked in all cases by an extensive search in the parameter space starting with different estimates. A convergence to a unique solution was always found. Optimization by point perturbation analysis as described elsewhere (Di Cera et al., 1991) was also carried out to check the convergence of the fitting algorithm to a unique point.

Control Experiments. The possibility of S-2238 being a "sticky" substrate, with the acylation rate being faster than dissociation, was thoroughly considered. Control experiments were carried over the entire pH range 6–10 under experimental conditions of 1 nM thrombin, 25 mM BisTris, 25 mM Tris, 25 mM CHES, 0.1 M NaCl and 0.1% PEG 8000, at 25 °C. Steady-state measurements were carried out with S-2238 and the amide substrate BzArgNHNp, a poor nonsticky substrate for thrombin for which K_m equals the equilibrium dissociation constant (Griffith, 1979). Measurements of BzArgNHNp hydrolysis were carried out as in the case of S-2238. At each pH, seven data points corresponding to different substrate concentrations were collected and analyzed according to eq 2 to yield the values of K_m and k_{cat}. The range of BzArgNHNp concentrations spanned in these experiments was from 40 μM to 2.5 mM. Analysis of the pH dependence of K_m and k_{cat} for BzArgNHNp yielded pK values for the free enzyme that are in perfect agreement with those obtained in the case of S-2238 (see Discussion and Table V), thereby implying that K_m for S-2238 is indeed equivalent to the equilibrium dissociation constant of the adduct. The quasi-equilibrium hypothesis dealt with in the treatment of the linkage scheme in eq 3 is thus fully justified. Additional control experiments were carried out over the entire pH range 6–10 under identical solution conditions with the competitive inhibitor *p*-aminobenzamidine (Mares-Guia & Shaw, 1965; Evans et al., 1982; De Cristofaro & Di Cera, 1991). At each pH, initial velocity measurements were collected in a 7 × 7 matrix, with seven different concentrations of S-2238 and seven different concentrations of *p*-aminobenzamidine, in the range from 10 to 500 μM, for each substrate concentration (De Cristofaro & Di Cera, 1991). At each concentration of *p*-aminobenzamidine, the data were analyzed according to eq 2 to yield the values of K_m and k_{cat}. The k_{cat} values were always found to be equal within experimental error (typically <2%), while K_m was found to increase linearly with the concentration [I] of *p*-aminobenzamidine, as expected for competitive inhibition (Fersht, 1985), according to the equation $K_m = K_m'(1 + [I]/K_I)$, where K_m' is the value of K_m in the absence of inhibitor and K_I is the dissociation constant of the inhibitor. The values of K_I obtained in this way over the entire pH range were then analyzed to extract the pK values of the ionizable groups controlling the binding of *p*-aminobenzamidine to thrombin. Analysis of the pH dependence of K_I for *p*-aminobenzamidine again yielded pK values for the free enzyme that are in perfect agreement with those obtained in the case of S-2238 (see Discussion and Table V). This shows unequivocally, on the basis of kinetic theory (Cleland, 1982), that S-2238 is not a sticky substrate and its K_m is equivalent to the equilibrium dissociation constant of the adduct.

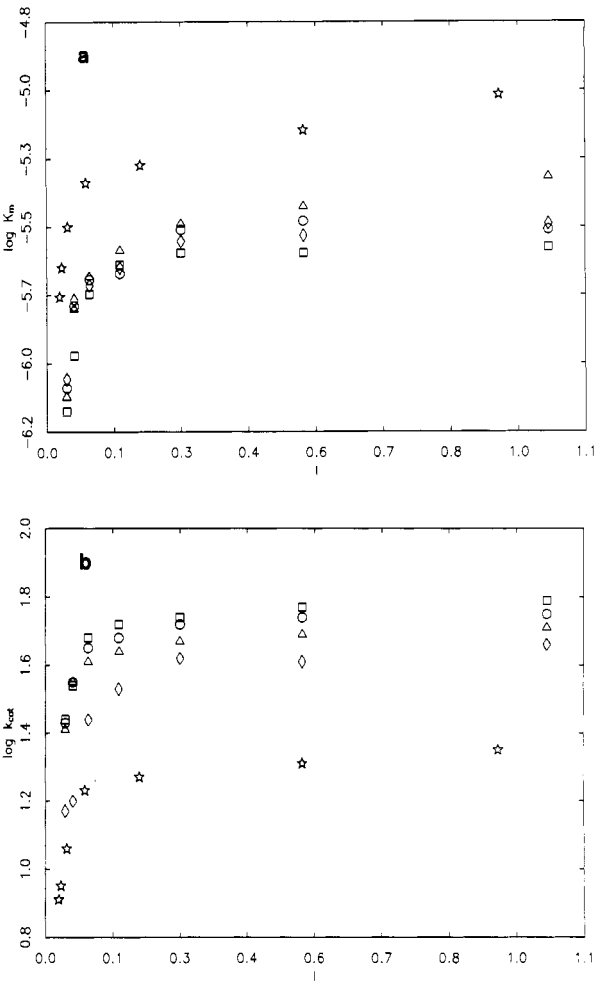


FIGURE 1: Effect of ionic strength on K_m (a) and k_{cat} (b) for human α-thrombin amidase activity, under experimental conditions of 50 mM Tris and 0.1% PEG 8000, at pH 8.00 and 25 °C. Salts are as follows: (□) NaCl; (○) NaBr; (△) NaI; (◇) KCl; (☆) $MgCl_2$.

Results

The contribution of ionic strength effects to catalytic and binding events in human α-thrombin can be tested by measuring the values of K_m and k_{cat} in the presence of different salts. Salt effects have long been subject to experimental investigation in protein and enzyme chemistry, but their theoretical bases are only incompletely understood (Edsall & Wyman, 1958; Tanford, 1962; von Hippel & Schleich, 1969). Aspecific ionic interactions are independent of the particular salt being used and depend solely on the ionic strength of the medium. When this is not the case, then specific binding interactions of the cationic and/or anionic components of the salt must be invoked. The strategy of studying ion effects with a number of salts sharing either the anionic or the cationic component

Table II: Effect of NaCl on the Parameters Involved in the Linkage Scheme in Eq 3[a]

	free enzyme			adduct		
[NaCl]	pK_1	pK_2	pK_3	pK_1	pK_2	pK_3
	Effect of NaCl Concentration (M) on the pK Values of the Ionizable Groups					
1.000	6.67 ± 0.22	8.45 ± 0.13	7.86 ± 0.28	6.14 ± 0.28	9.03 ± 0.12	7.86 ± 0.28
0.333	6.74 ± 0.35	8.28 ± 0.23	8.12 ± 0.43	6.17 ± 0.41	8.86 ± 0.19	8.12 ± 0.43
0.111	6.71 ± 0.33	8.73 ± 0.22	8.00 ± 0.42	6.12 ± 0.43	9.36 ± 0.23	8.00 ± 0.43
0.037	6.99 ± 0.34	8.51 ± 0.20	8.22 ± 0.36	6.29 ± 0.40	9.61 ± 0.21	8.22 ± 0.36
0.012	7.14 ± 0.27	8.43 ± 0.16	8.40 ± 0.17	6.44 ± 0.27	9.84 ± 0.17	8.40 ± 0.27
0.004	7.25 ± 0.20	8.48 ± 0.12	8.56 ± 0.12	6.55 ± 0.19	10.02 ± 0.16	8.56 ± 0.12
0.001	7.01 ± 0.13	8.59 ± 0.06	8.58 ± 0.07	6.36 ± 0.16	10.10 ± 0.07	8.58 ± 0.07
[NaCl]	$^0k_2$	$^1k_2$	$^2k_2$	$^3k_2$	^0K_d	σ
	Effect of NaCl Concentration (M) on the Acylation Rates (s^{-1}) and the Substrate Dissociation Constant ^0K_d (μM)					
1.000	36.3 ± 2.7	68.2 ± 3.3	32.3 ± 3.1	24.3 ± 3.3	7.64 ± 0.48	0.025
0.333	35.4 ± 3.9	69.7 ± 4.9	31.6 ± 4.3	21.2 ± 5.0	6.81 ± 0.59	0.039
0.111	29.6 ± 6.0	58.6 ± 8.1	26.6 ± 8.0	18.6 ± 8.4	6.61 ± 0.53	0.047
0.037	28.2 ± 9.7	52.4 ± 14.5	14.9 ± 8.6	12.4 ± 9.1	7.16 ± 0.74	0.060
0.012	20.0 ± 10.6	54.8 ± 15.6	6.6 ± 4.2	4.0 ± 2.1	8.30 ± 1.00	0.047
0.004	8.7 ± 6.3	57.4 ± 13.2	5.3 ± 3.8	2.1 ± 1.7	9.23 ± 1.12	0.036
0.001	5.5 ± 2.2	56.6 ± 12.4	3.7 ± 2.3	1.5 ± 1.2	9.82 ± 0.63	0.034

[a] Errors are at the cutoff of one standard deviation. [b] The standard deviation of the fit, σ, is given in log units.

may be revealing of aspecific and specific interactions (Lohman, 1985). The results obtained for human α-thrombin are shown in Figure 1. It is clear that the effects observed on the values of the Michaelis–Menten parameters are strongly dependent on the particular salt being used. This result is in agreement with a previous study of the effects of salts on human α-thrombin clotting and esterase activity (Landis et al., 1981) and shows unequivocally that ionic strength effects play only a secondary role in controlling thrombin activity. Human α-thrombin is capable of efficiently discriminating among efficient cations as well as anions. This strongly supports the idea that small ions may influence thrombin activity by specifically binding to the enzyme. The terms reflecting specific binding interactions in eq 11 are thus likely to overwhelm the last two terms on the right-hand side arising from aspecific ionic interactions. Also, the term arising from differential hydration can be neglected under the experimental conditions employed in this study, since it can only influence the results at very high (≫0.5 M) salt concentrations (Record et al., 1978), and one is left only with the specific differential binding of the cationic and anionic components of the salt. In practice, the effect of NaCl on thrombin amidase activity can be cast in terms of specific binding interactions of Na^+ and Cl^- ions with the enzyme.

The effect of NaCl on the pH dependence of human α-thrombin amidase activity is shown in Figure 2, and the best-fit values of the parameters involved in the linkage scheme are listed in Table II. The pH dependence of K_m shows a typical bell-shaped curve as found under different buffer conditions (De Cristofaro & Di Cera, 1990) and in the case of other serine proteases (Fersht, 1985). The pH dependence of k_{cat} is also bell shaped, although the effect is less evident, with a maximum around pH 9. Among the three ionizable residues responsible for the pH dependence of the Michaelis–Menten parameters, two are involved in the control of K_m (pK_1 and pK_2 in Table II) and change their pK upon substrate binding to the enzyme. The third group (pK_3 in Table II) shows no significant change in pK with substrate binding and hence does not affect the value of K_m but shares with the other two groups the control of k_{cat}. Due to the bell-shaped pH dependence of K_m, protons are released upon substrate binding at pH < 7.5 and taken up at higher pH values. The maximum linkage between proton and substrate binding to human α-thrombin is derived from eq 7 and is 0.32 protons released per substrate bound at pH ≈ 6.4 and 0.32 protons uptaken per substrate bound at pH ≈ 8.7. It should be noted that this linkage is smaller than that found under different conditions where phosphate was used as buffer and four ionizable groups were needed to adequately fit the data (De Cristofaro & Di Cera,

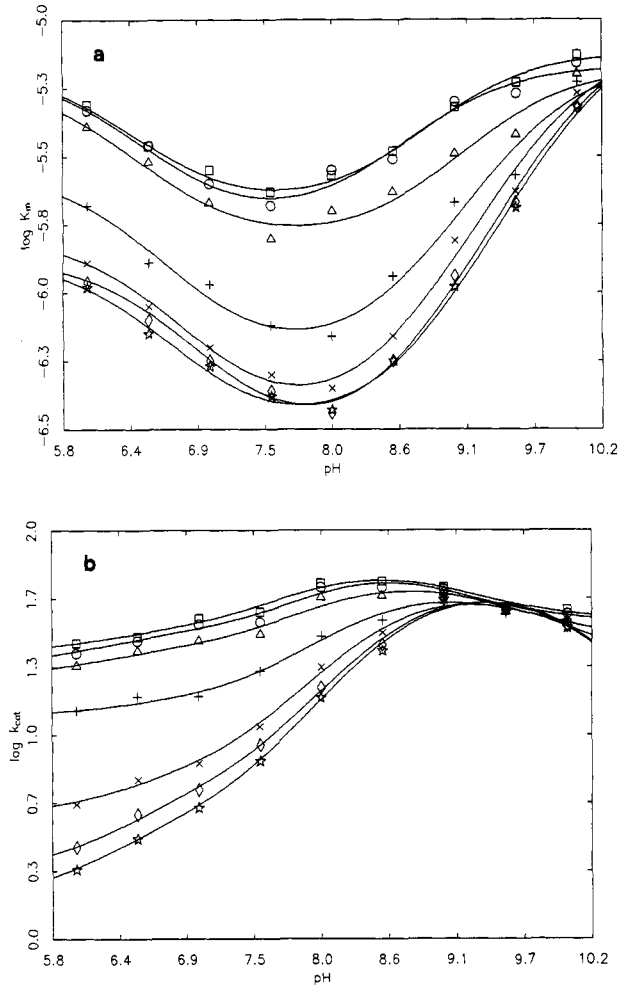


FIGURE 2: Results of the pH dependence of the Michaelis–Menten parameters K_m (a) and k_{cat} (b) for human α-thrombin amidase activity, as a function of NaCl. Points are best-fit values obtained from analysis of experimental data with eq 2. Continuous lines were drawn from eqs 19 and 20 by using the parameter values listed in Table II. The concentration of NaCl is as follows: (□) 1.000 M; (○) 0.333 M; (△) 0.111 M; (+) 0.037 M; (×) 0.012 M; (◇) 0.004 M; (☆) 0.001 M.

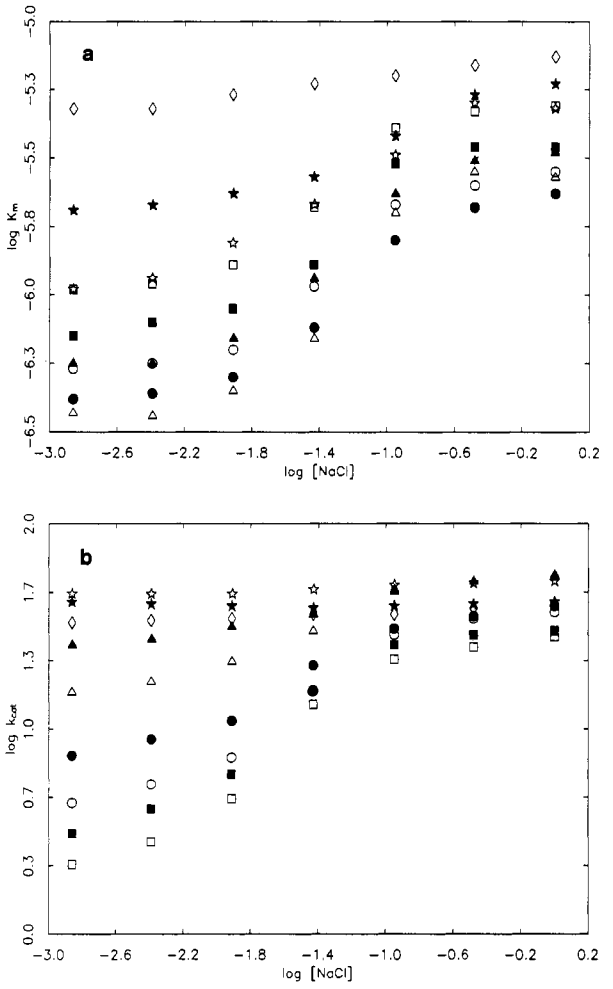


FIGURE 3: Results of the NaCl dependence of the Michaelis-Menten parameters K_m (a) and k_{cat} (b) for human α-thrombin amidase activity, as a function of pH. The data are the same as those shown in Figure 2 but plotted as a function of NaCl to demonstrate how the effect of the salt saturates out at both low and high concentrations, which is suggestive of specific ion binding interactions of Na^+ and Cl^- with the enzyme. The pH values are as follows: (□) 6.00; (■) 6.50; (○) 7.00; (●) 7.50; (△) 8.00; (▲) 8.50; (☆) 9.00; (★) 9.50; (◇) 10.00.

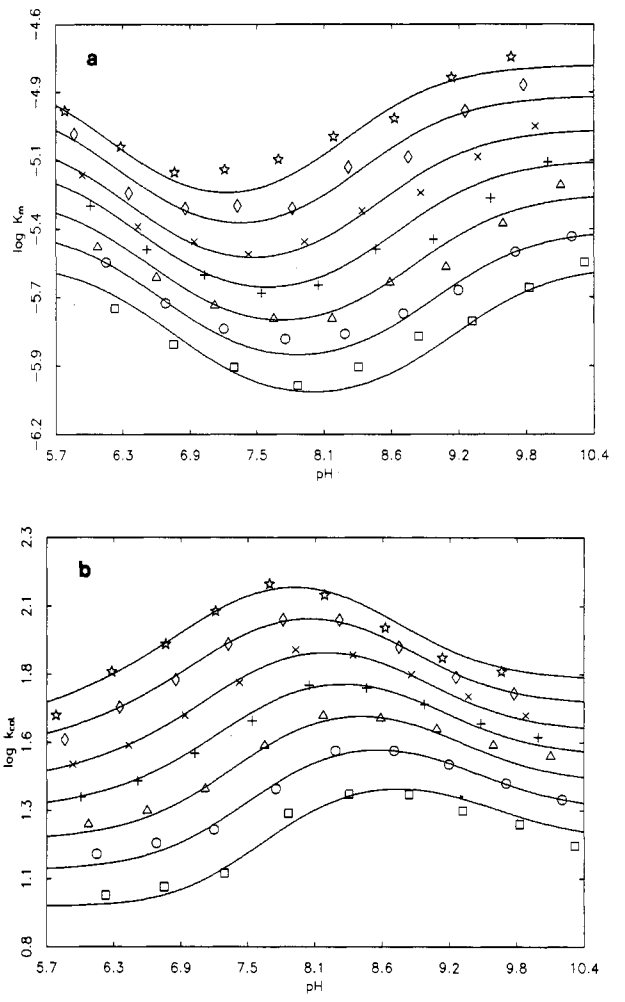


FIGURE 4: Results of the pH dependence of the Michaelis-Menten parameters K_m (a) and k_{cat} (b) for human α-thrombin amidase activity, as a function of temperature. Points are best-fit values obtained from analysis of experimental data with eq 2. The pH values at all temperatures were calculated by using the $\Delta pH/\Delta T$ coefficients reported in Table I. Continuous lines were drawn from eqs 21–25 by using the parameter values listed in Table III. The temperature values are as follows: (□) 10 °C; (○) 15 °C; (△) 20 °C; (+) 25 °C; (×) 30 °C; (◇) 35 °C; (☆) 40 °C.

1990). However, this is not surprising in view of the sensitivity of ionization reactions to buffer conditions, a prototypic example of which is the Bohr effect of human hemoglobin (Russu & Ho, 1986). Also, since anionic effectors such as adenosine nucleotides have been reported to affect thrombin activity (De Cristofaro et al., 1990), a possible interaction of phosphate itself with the enzyme cannot be ruled out. Although the use of a BisTris/Tris/CHES buffer seems to have eliminated these (possible) complicating effects, the main features of the pH dependence of thrombin amidase activity, i.e., the bell-shaped dependence of K_m and the multiple inflection points of the dependence of k_{cat}, remain qualitatively unaltered. In particular, the pK values of the ionizable groups controlling K_m in the free enzyme are identical, within errors, with those obtained in phosphate buffer. Also, a most important feature of this linkage, namely, that the catalytic rate of the enzyme is more sensitive to changes in NaCl at low pH, is in perfect agreement with what is seen in phosphate buffer. The study of the pH dependence of the relevant kinetic parameters over a wide range of NaCl concentrations makes it possible to explore the linkage properties of the system also as a function of salt. This was not possible in a previous study where only four concentrations of salt have been examined (De Cristofaro & Di Cera, 1990). The NaCl dependence of K_m and k_{cat} is shown in Figure 3. Both parameters increase with increasing salt concentration, and the striking observation is that the effect of NaCl saturates out at both low and high concentrations. Such a dependence is typically observed in linkage effects involving specific binding interactions and strongly supports, in a completely independent fashion, the conclusion drawn from ion effects studies (see Figure 1) that Na^+ and Cl^- specifically interact with the enzyme and change its catalytic properties. Further support for this conclusion comes from the NaCl dependence of the pK values listed in Table II. These values are either unaffected or decrease with increasing NaCl. Since the effect of ionic strength is to stabilize charged residues, increasing ionic strength should decrease the pK of carboxyl groups and increase the pK of amino and imidazole groups (Tanford, 1962). None of the pK values in Table II is typical of carboxyl residues, yet they either decrease or remain unchanged with increasing NaCl, contrary to the expectation drawn from consideration of ionic strength effects. All these findings provide overwhelming evidence that small ions indeed play an important role in controlling thrombin catalytic activity and that the effect of NaCl on thrombin amidase activity is specific and mediated by binding of Na^+ and Cl^- to the enzyme.

Table III: Best-Fit Values of the Parameters Involved in the Linkage Scheme in Eq 3 and Corresponding van't Hoff Enthalpies and Activation Energies[a]

Thermodynamic Parameters for Substrate and Proton Binding[b]		
	$^0K_d^0 = 7.37 \pm 0.36$	$^0\Delta H_s = -10.51 \pm 0.62$
	$pK_1 = 6.75 \pm 0.26$	$^E\Delta H_1 = 6.97 \pm 0.87$
free enzyme	$pK_2 = 8.36 \pm 0.35$	$^E\Delta H_2 = 13.33 \pm 1.38$
	$pK_3 = 7.51 \pm 0.29$	$^E\Delta H_3 = 8.89 \pm 1.16$
	$pK_1 = 6.16 \pm 0.25$	$^{ES}\Delta H_1 = 7.46 \pm 0.91$
adduct	$pK_2 = 8.95 \pm 0.41$	$^{ES}\Delta H_2 = 12.50 \pm 1.14$
	$pK_3 = 7.51 \pm 0.29$	$^{ES}\Delta H_3 = 8.89 \pm 1.16$
Thermodynamic Parameters for Catalysis[c]		
	$^0k_2^0 = 31.5 \pm 2.4$	$\Delta E_0 = 7.96 \pm 0.74$
	$^1k_2^0 = 69.6 \pm 5.9$	$\Delta E_1 = 10.27 \pm 1.22$
	$^2k_2^0 = 24.3 \pm 2.9$	$\Delta E_2 = 11.14 \pm 1.34$
	$^3k_2^0 = 19.8 \pm 2.5$	$\Delta E_3 = 8.95 \pm 1.46$

[a] Derived from global analysis of the data shown in Figure 4 according to eqs 21–25 in the text. Errors are at the cutoff of one standard deviation. [b] The substrate dissociation constant is in μM, while standard enthalpy values are in kcal/mol. pK values and $^0K_d^0$ are standard values calculated at 0.1 M NaCl and 0.1% PEG 8000, at 25 °C, under the buffer conditions given in the text. [c] The acylation rates are in s^{-1}, while energies of activation are in kcal/mol. k_2^0 values are standard values calculated at 0.1 M NaCl and 0.1% PEG 8000, at 25 °C, under the buffer conditions given in the text.

The effect of temperature on the pH dependence of human α-thrombin amidase activity is shown in Figure 4, and the best-fit values of the parameters involved in the linkage scheme are listed in Table III. The linkage scheme in eq 3 with the parameters expressed in terms of their temperature dependence (eqs 21–25) gives a very good fit of the data ($\sigma = 0.037$) and allows for resolution of all relevant enthalpic components to binding and catalytic events. The parameter values computed at the standard state (25 °C) are in nice agreement with the results of the NaCl studies, thereby showing the overall consistency of the experimental data reported in this study. The values of ionization enthalpies of the proton-linked ionizable groups are all very reasonable and lie within the range expected for imidazole and amino groups (Edsall & Wyman, 1958). The advantage of defininining K_m and k_{cat} in terms of detailed partition functions becomes obvious when it comes to computing all thermodynamic quantities characterizing substrate binding and catalysis. The free energy of binding is in fact given by

$$\Delta G_s = RT \ln K_m \quad (26)$$

and the enthalpy of binding as a function of pH is given by the Gibbs–Helmholtz equation applied to eqs 21–25, i.e.,

$$\Delta H_s = \partial[\Delta G_s(h)/T]/\partial\tau = {}^0\Delta H_s + R\,\partial \ln Z_E(T)/\partial\tau - R\,\partial \ln Z_{ES}(T)/\partial\tau = {}^0\Delta H_s + {}^E\Delta H(h) - {}^{ES}\Delta H(h) \quad (27)$$

The above expression shows that the enthalpy of substrate binding at any given pH (in the range 6–10) equals the standard enthalpy value in the absence of protons, $^0\Delta H_s$, plus the difference between two pH-dependent terms, namely, the overall enthalpies of ionization of the free enzyme and the adduct. The last two terms in the right-hand side of eq 27 give the enthalpic contribution to substrate binding arising from proton dissociation. The thermodynamic dissection of the free energy of substrate binding into its enthalpic and entropic components as a function of pH is given in Table IV. It is of interest to note that ΔH_s is practically temperature independent, as obtained by application of eq 27. This result is particularly important. In fact, a plot of the logarithm of K_m versus $1/T$ is not expected to be linear in general, since K_m may be a nonlinear function of various van't Hoff and Arrhenius terms. However, if substrate dissociation is faster

Table IV: Thermodynamic Parameters for Substrate Binding and Catalysis as a Function of pH[a]

pH	ΔG_s	ΔH_s	$T\Delta S_s$	ΔE
6.00	−7.23 ± 0.14	−8.22 ± 0.49	−1.00 ± 0.51	10.68 ± 0.64
6.50	−7.43 ± 0.15	−7.70 ± 0.46	−0.27 ± 0.49	11.87 ± 0.71
7.00	−7.61 ± 0.15	−8.54 ± 0.51	−0.92 ± 0.53	12.84 ± 0.77
7.50	−7.69 ± 0.15	−10.14 ± 0.61	−2.45 ± 0.63	12.59 ± 0.76
8.00	−7.64 ± 0.15	−12.21 ± 0.73	−4.56 ± 0.75	11.04 ± 0.66
8.50	−7.48 ± 0.15	−14.05 ± 0.84	−6.57 ± 0.86	9.09 ± 0.55
9.00	−7.27 ± 0.15	−13.89 ± 0.83	−6.62 ± 0.85	7.47 ± 0.45
9.50	−7.12 ± 0.14	−12.35 ± 0.74	−5.23 ± 0.75	6.97 ± 0.42
10.00	−7.05 ± 0.14	−11.23 ± 0.67	−4.18 ± 0.69	7.33 ± 0.44

[a] Under experimental conditions of 0.1 M NaCl and 0.1% PEG 8000, at 25 °C. All values are given in kcal/mol, and errors are at the cutoff of one standard deviation.

compared to catalysis, then K_m approximates the dissociation constant of the adduct, and a plot of the logarithm of K_m versus $1/T$ is indeed linear (Laidler, 1969; Laidler & Peterman, 1979). The temperature independence of ΔH_s thus provides independent and strong evidence that indeed substrate dissociation is faster than acylation over the entire pH and temperature ranges analyzed in this study. Analogous considerations apply to the case of the energy of activation

$$\Delta E = -R\,\partial \ln k_{cat}/\partial\tau = R\,\partial \ln Z_{ES}(T)/\partial\tau - R\,\partial \ln P_{ES}(T)/\partial\tau = {}^{ES}\Delta H(h) - {}^{ES}\Delta H^*(h) \quad (28)$$

that at any given pH equals the difference between the overall enthalpy of ionization of the adduct and an enthalpy term that reflects the contribution of the activation energies and ionization enthalpies of all protonated intermediates of the adduct. The values of ΔE are given in Table IV. They are almost temperature independent and nicely agree with activation energy values obtained for the hydrolysis of amide substrates by chymotrypsin and trypsin (Butler, 1941). This result too is important as it suggests that acylation is indeed rate limiting over the pH and temperature ranges explored in this study.

Discussion

There have been no systematic and quantitative studies on the pH dependence of human α-thrombin amidase activity over a wide range of NaCl concentrations and temperatures. Previous studies of the effect of salts on thrombin activity have not explored a wide pH range and have yielded conflicting results. One study has reported that increasing NaCl concentration decreases thrombin activity (Workman & Lundblad, 1978). Another study has shown just the opposite, with k_{cat} increasing and K_m decreasing with NaCl concentration (Orthner & Kosow, 1980). Such a drastic discrepancy is difficult to rationalize. The results presented here show that increasing NaCl concentration increases both k_{cat} and K_m and that this effect is observed over the pH range 6–10. It is evident that the information collected in studies conducted over a wide range of experimental conditions is absolutely critical to correctly assess the energetic nature of the structural perturbations that translate into the underlying binding and catalytic events. Also, these studies provide a reliable data base that can be used to map the energetics of the enzyme for each linked effect. A detailed and quantitative understanding of the complex functional and regulatory aspects of the thrombin system can only be accomplished by thorough experimental investigation of the properties of this enzyme under a variety of solution conditions. Since its catalytic mechanism is identical with that of other serine proteases, it is clear that the bases of thrombin's peculiar functional features must be looked for not in the catalytic mechanism itself but rather in

the way this mechanism is controlled and regulated by chemical, biochemical, and physical linkage effects.

Analysis of the pH dependence of thrombin amidase activity is a critical step in the foregoing experimental strategy, since the proton is a ubiquitous driving force for biochemical processes. The linkage with protons often reveals the macromolecular structural components involved in binding or catalytic events and helps to identify the thermodynamic bases for the changes of structural and functional significance. We have found that at least three ionizable groups should be taken into account in the control of thrombin amidase activity. This feature seems to be unique for thrombin, since other serine proteases show a pH dependence of their activity that can be accounted for by only two ionizable residues (Renard & Fersht, 1973; Fersht & Renard, 1974; Fersht, 1985). Under experimental conditions of 0.1 M NaCl and 25 °C, one of the three groups has a pK of 6.75 ± 0.26 in the free enzyme that changes to 6.16 ± 0.25 in the adduct. These values are practically identical with those found for the His of the active site in chymotrypsin (Fersht, 1985). The standard enthalpy of ionization of this residue is 6.97 ± 0.87 kcal/mol in the free enzyme and 7.46 ± 0.91 kcal/mol in the adduct. These values are typical of an imidazole group (Edsall & Wyman, 1958; Tanford, 1962) and support the idea that one of the three residues involved in the control of thrombin amidase activity may indeed be the active site His. The second group has a pK of 8.36 ± 0.35 in the free enzyme and 8.95 ± 0.41 in the adduct. The standard enthalpy of ionization of this group is 13.33 ± 1.38 kcal/mol in the free enzyme and 12.50 ± 1.14 kcal/mol in the adduct. These values suggest involvement of an amino group. In analogy with chymotrypsin and trypsin, one can assign these values to the amino terminus (Fersht, 1985), which would make two of the three groups controlling thrombin catalytic activity identical with those found in other serine proteases. The third group does not change its pK upon substrate binding, and therefore it controls k_{cat} but not K_m. Since such a group is not involved in the control of the enzymatic activity of other serine proteases, it is tempting to speculate that it might be located in a thrombin structural domain that has no counterpart in the serine protease family. A domain having such a property is the anion-binding exosite located on top of the groove extension leading to the catalytic pocket (Bode et al., 1989). The anion-binding exosite is characterized by a patch of basic residues that are important in the interaction with fibrinogen (Fenton, 1986; Lewis et al., 1987; Fenton et al., 1988; Vali & Sheraga, 1988), hirudin (Fenton & Bing, 1986; Stone & Hofsteenge, 1986; Stone et al., 1987), thrombomodulin (Tsiang et al., 1990), and a number of allosteric effectors (Conery & Berliner, 1986; De Cristofaro et al., 1990). It will be of interest to test the hypothesis that the third ionizable group actually belongs to the anion-binding exosite by measuring the pH dependence of amidase activity in human γ-thrombin, where the regulatory site is drastically perturbed. These studies, that are now underway in our laboratory, will provide an ideal case for application of the recently developed theory of "contracted partition functions" (Di Cera, 1990), by which structural perturbations can be used to access site-specific thermodynamic properties.

It is important to stress that the pK values reported in this study reflect proton binding reactions and are not distorted by the influence of kinetic rates, as it would be the case if S-2238 were a sticky substrate (Cleland, 1982). In fact, if the acylation rate k_2 is faster than the dissociation rate k_{-1}, then K_m equals k_2/k_1^* and is a function not only of proton

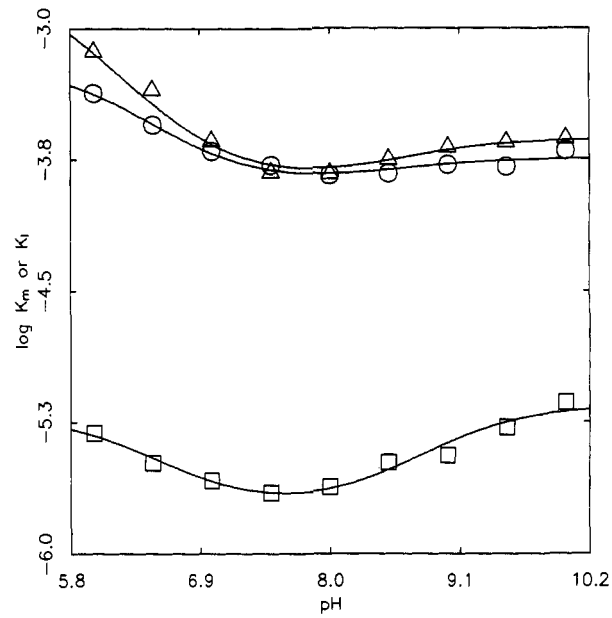


FIGURE 5: Results of the pH dependence of the Michaelis–Menten parameter K_m for S-2238 (□) and BzArgNHNp (△) and of K_I for *p*-aminobenzamidine (○). Continuous lines were drawn from eq 19 with $\alpha_3 = \beta_3$, by using the best-fit parameter values listed in Table V (top).

binding constants but also of several kinetic rates (Cleland, 1982; De Cristofaro & Di Cera, 1990). The possibility of S-2238 being a sticky substrate has carefully been considered and ruled out as follows. If K_m for S-2238 is a dissociation constant for the substrate, then the pH dependence of K_m yields pK values for the free enzyme that must be identical with those found from the pH dependence of K_m of other substrates that bind with the same mechanism. Furthermore, the pH dependence of K_I for a competitive inhibitor must also yield the same pK values for the free enzyme (Cleland, 1982). The pK values of the adduct will of course be dependent on the particular substrate or inhibitor being used and need not be identical. The pH dependence of K_m for a BzArgNHNp and K_I for *p*-aminobenzamidine are shown in Figure 5, along with the pH dependence of K_m for S-2238 under identical solution conditions. The amide substrate BzArgNHNp is certainly not sticky since it has a k_{cat} value 500 times smaller than that of S-2238 and a K_m 100 times bigger (Griffith, 1979), while the K_I of the competitive inhibitor *p*-aminobenzamidine reflects exclusively binding reactions. The data in Figure 5 were analyzed altogether by using eq 19 for either K_m or K_I. Since only two groups control the pH dependence of K_m, then $\alpha_3 = \beta_3$ (see also Tables II and III) and these two parameters can be dropped from the analysis. The same values of α_1 and α_2, reflecting the protonation reactions of the two groups of the free enzyme, were used for all three data sets shown in Figure 5, while β_1 and β_2 were allowed to be different since they reflect protonation reactions of the adduct. The excellent fit of the three data sets shown in Figure 5 by continuous lines demonstrate that S-2238, BzArgNHNp, and *p*-aminobenzamidine are probing the same protonation reactions and therefore S-2238 is not a sticky substrate. The pK values listed in Table V (top) summarize the energetics of these reactions. The pK values of the free enzyme are in very good agreement with those obtained from the temperature studies with S-2238 (see Table III). The three data sets were also analyzed separately, and the results are given in Table V (bottom). Again, an excellent agreement is found among the pK values of the free enzyme obtained in the three cases. Finally, since the values

Table V: Best-Fit Values of the Parameters Involved in the pH Dependence of K_m and K_I (in μM) for the Data Shown in Figure 5[a]

	free enzyme	adduct with S-2238	adduct with BzArgNHNp	adduct with *p*-aminobenzamidine
Simultaneous Analysis Based on Eq 19 in the Text with Two Ionizable Groups ($\alpha_3 = \beta_3$)				
pK_1	6.83 ± 0.16	6.30 ± 0.11	5.64 ± 0.25	6.15 ± 0.18
pK_2	8.46 ± 0.20	9.04 ± 0.30	8.70 ± 0.15	8.60 ± 0.19
${}^0K_d$		6.93 ± 0.35	233.0 ± 30.7	
${}^0K_I$				180.7 ± 23.5

	with S-2238		with BzArgNHNp		with *p*-aminobenzamidine	
	free enzyme	adduct	free enzyme	adduct	free enzyme	adduct
Individual Analysis Based on Eq 19 in the Text with Two Ionizable Groups ($\alpha_3 = \beta_3$)[b]						
pK_1	6.77 ± 0.14	6.22 ± 0.12	6.91 ± 0.18	5.55 ± 0.22	6.89 ± 0.15	6.23 ± 0.17
			(6.82 ± 0.22)	(5.80 ± 0.25)	(6.79 ± 0.20)	(6.25 ± 0.16)
pK_2	8.45 ± 0.21	9.05 ± 0.20	8.41 ± 0.22	8.61 ± 0.29	8.52 ± 0.20	8.67 ± 0.35
${}^0K_d$	6.86 ± 0.45		233.4 ± 32.2			
			(191.6 ± 46.4)			
${}^0K_I$					183.0 ± 19.1	
					(167.1 ± 27.1)	

[a] Errors are at the cutoff of one standard deviation. [b] The best-fit values in parentheses refer to the results of individual analysis based on eq 19 with one ionizable group ($\alpha_2 = \beta_2$; $\alpha_3 = \beta_3$).

of pK_2 for BzArgNHNp and *p*-aminobenzamidine turned out to be almost the same for both the free enzyme and adduct, the pH dependence of K_m for BzArgNHNp and K_I for *p*-aminobenzamidine was also analyzed in terms of a single ionizable group. Although the fit with a single ionizable group was significantly worse than that with two ionizable groups, nevertheless the values of pK_1 for the free enzyme agree very well with those determined in all other analyses and are given in Table V (bottom) in parentheses. In view of this substantial evidence obtained independently with a poor substrate and a competitive inhibitor, with the experimental data being analyzed in a variety of ways, it can be concluded with high confidence that (1) S-2238 is not a sticky substrate; (2) the pK values obtained with S-2238 are indeed reflections of ionization reactions underlying substrate binding and catalysis; and (3) the quasi-equilibrium hypothesis dealt with in the mathematical analysis of the linkage scheme in eq 3 is correct. Additional and independent support to these conclusions comes from previous studies on bovin thrombin. It has been reported that amide substrates with widely different k_{cat}/K_m values, including one with a ratio comparable to that of S-2238, show the same pH dependence of K_m (Lottenberg et al., 1983). This dependence is also similar to that obtained with an ester substrate for which acylation is rate limiting and dissociation is faster than acylation (Ascenzi et al., 1982).

The effect of small ions reported in this study further supports the idea of a central role played by the anion-binding exosite in this enzyme. Thrombin is capable of discriminating among different ions, and ionic strength effects have very little influence on its enzymatic properties. We have not attempted to formulate a canonical partition function for the thrombin system in terms of detailed Na^+ and Cl^- binding sites, although the data shown in Figure 3 can easily be fitted by linkage equations containing two ion-binding sites. More information on the properties of the enzyme in the presence of different salts (other than NaCl) over the entire pH range from 6 to 10 is needed to construct a detailed partition function for the system. In particular, the separate contribution of each cation and anion needs to be addressed experimentally. Combined experiments with NaBr, KCl, and KBr, along with the results reported here, should help to clarify the differential interactions of cations for a given anion and vice versa, which can be used to develop a statistical thermodynamic model of Na^+ and Cl^- binding to thrombin. Extension of this strategy to the study of γ-thrombin may be revealing of the role of the anion-binding exosite in small ion binding.

The information collected from temperature studies of the pH dependence of thrombin amidase activity complements the thermodynamic picture derived from ion effects and provides details on the enthalpic and entropic components of binding and catalytic events. Also, it yields additional information on the ionizable groups that affect binding and catalysis and provides an independent check of the assumptions made in the derivation of eq 3 and eqs 19 and 20. We have shown that the linkage scheme in eq 3 is perfectly consistent with the results obtained experimentally when all parameters are formulated in terms of simple van't Hoff and Arrhenius expressions. The internal consistency of experimental data, in conjunction with the theoretical approach based on eq 3 and powerful computational procedures, have altogether made it possible to resolve all parameters involved in the linkage scheme.

Functional properties approached in terms of linkage thermodynamics provide a great deal of information to be exploited from analysis of experimental data. We have shown how the strategy of generating, analyzing and interpreting this information can successfully be used in the study of the thrombin system when each step of the experimental, theoretical, and computational approaches is given careful consideration. Extension of the same strategy to the study of linkage effects in structurally perturbed thrombin derivatives is now underway in our laboratory. These studies will provide key information on the energetic aspects of the linkage between functional properties and structural perturbations in the thrombin system.

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