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# Linkage between Proton Binding and Amidase Activity in Human γ-Thrombin†

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ABSTRACT: The amidase activity of human γ-thrombin has been studied in the pH range 6–10 as a function of NaCl concentration and temperature. As recently found for human α-thrombin [Di Cera, E., De Cristofaro, R., Albright, D. J., & Fenton, J. W., II (1991) *Biochemistry 30*, 7913–7924], the Michaelis–Menten constant, $K_m$, shows a bell-shaped dependence over this pH range with a minimum around pH 7.9 in the presence of 0.1 M NaCl at 25 °C. The catalytic constant, $k_{cat}$, has a bell-shaped pH dependence with a maximum around pH 8.6. A thermodynamic analysis of these parameters has enabled a characterization of the linkage between proton and substrate binding, its dependence on NaCl concentration, and the relevant entropic and enthalpic contributions to binding and catalytic events. Three groups seem to be responsible for the control of γ-thrombin amidase activity as a function of pH. One of these groups has p$K$ values that are significantly different from those found for α-thrombin, and all groups show slightly perturbed enthalpies of ionization. The dependence of γ-thrombin amidase activity on NaCl concentration is different from that of α-thrombin. Increasing NaCl concentration always decreases the substrate affinity for the enzyme in the case of α-thrombin, regardless of pH. In the case of γ-thrombin, such an effect is observed only in the pH range 7.5–9, and a reversed linkage is observed at pH <7 and >9.5. It is proposed that the perturbed functional properties of γ-thrombin compared with the native enzyme are due to structural perturbations of the anion binding exosite for the recognition of fibrinogen.

Human thrombin, the enzyme that catalyzes the critical reaction leading to clot formation in the coagulation cascade, is a member of the large family of serine proteases. Although its catalytic mechanism does not differ from that of other serine proteases, the way thrombin amidase activity is affected by salts, pH, and temperature is unusual. The thermodynamic bases of this behavior have recently been explored in a quantitative way (De Cristofaro & Di Cera, 1990; Di Cera et al., 1991) and can now be related to the underlying structural features of the enzyme (Bode et al., 1989). A natural question that arises in this connection is how the insertion loops contribute to thrombin's unique regulatory properties and

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functional specificity. One of these loops, the anion binding exosite (ABE), is important for fibrinogen recognition and has long been suggested to play a fundamental role in the modulation of thrombin activity (Fenton, 1986; Fenton et al., 1988). A thorough understanding of the role of this important regulatory site can only be accomplished by quantitative studies of thrombin activity carried out on a number of enzyme derivatives that are perturbed at the level of the ABE. Gross structural perturbations of the ABE can be obtained by enzymatic digestion of thrombin with trypsin (Bing et al., 1977; Braun et al., 1988; Brezniak et al., 1990), which yields the derivatives $\beta$- and $\gamma$-thrombin. These derivatives have little or no clotting activity, but retain amidase activity toward small synthetic substrates (Conery & Berliner, 1983; Hofsteenge et al., 1988). Chemical modifications of important residues of the ABE leading to analogous effects have also been reported (Griffith, 1979; Chang, 1989; Church et al., 1989). In this paper, we begin our investigation of the effect of structural perturbations on thrombin's functional properties by studying the properties of human $\gamma$-thrombin. We follow the same strategy recently applied in the case of human $\alpha$-thrombin (De Cristofaro & Di Cera, 1990; Di Cera et al., 1991). The linkage between proton binding and amidase activity is studied as a function of NaCl concentration and temperature, using a combination of theoretical, computational, and experimental approaches to characterize the energetics of the reactions involved in the linkage scheme.

## Materials and Methods

The important thermodynamic aspects that are relevant to the analysis of the linkage effects reported in this study have been dealt with in detail elsewhere (De Cristofaro & Di Cera, 1990; Di Cera et al., 1991). All linkage equations used in the analysis of the experimental data reported here can be found in the paper by Di Cera et al. (1991).

*Thrombin Preparation.* Human $\gamma$-thrombin was prepared as described elsewhere (Bing et al., 1977; Brezniak et al., 1990). The preparation was 99% pure as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] on 5–25% gradient gels and silver staining. Thrombin concentration was measured using an extinction coefficient $E_{280}$ = 1.83 mL mg$^{-1}$ cm$^{-1}$ and a molecular weight of 36 500 (Fenton et al., 1977). The active-site concentration determined by titration with *p*-nitrophenylguanidinobenzoate hydrochloride (Chase & Shaw, 1967) was 84% of that determined spectrophotometrically and provided the value used in the calculation of $k_{cat}$ from analysis of steady-state measurements. Thrombin solutions of 2 $\mu$M concentration were stored in 50-$\mu$L vials at −80 °C until use.

*Steady-State Measurements.* Steady-state measurements of human $\gamma$-thrombin amidase activity were made using the synthetic chromogenic peptide S-2238 as described in detail elsewhere (Di Cera et al., 1991). The pH dependence of thrombin amidase activity was studied in the pH range 6–10 using a triple buffer containing 25 mM BisTris, 25 mM Tris, and 25 mM CHES. Addition of 0.1% PEG 8000 under all solution conditions was to prevent absorption of the enzyme to the cuvette walls. The concentration of released *p*-nitroaniline was quantified by means of an extinction coefficient derived from the empirical expression:

$$E_{405} = 9789.1 + 1334.6I/(2.0 + I) \quad (1)$$

where $I$ is the ionic strength of the solution. The expression above was derived from analysis of data obtained in triplicate under experimental conditions widely used in the linkage studies reported here, i.e., 25 mM BisTris, 25 mM Tris, 25 mM CHES, 0.1% PEG 8000, pH 8.0, and 25 °C. The ionic strength was changed by addition of NaCl to the buffer. A range of ionic strength values from 0.035 to 2 M was explored. Extinction coefficients for *p*-nitroaniline measured in a previous study (Lottenberg & Jackson, 1983), under slightly different experimental conditions, were previously fitted to the straight line $E_{405}$ = 9783.6 + 596.5$I$ (Di Cera et al., 1991). This expression does not differ significantly from eq 1 in the ionic strength range explored here and in the previous study. At higher ionic strength, the difference becomes significant, and eq 1 should be used. Extinction coefficients for *p*-nitroaniline change very little with pH and temperature. Steady-state measurements of substrate hydrolysis were analyzed according to the equation:

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

In the case of thrombin amidase activity, $e_T$ is the active-site concentration, $k_{cat}$ is the rate-limiting acylation, and $K_m = k_{-1}/k_1^* \equiv K_d$ is the dissociation constant of the substrate. The kinetic parameters $K_m$ and $k_{cat}$ were obtained by nonlinear least-squares as detailed elsewhere (Di Cera et al., 1991). The catalytic activity of human $\gamma$-thrombin was explored over the pH range 6–10 as a function of temperature and NaCl concentration. The concentrations of NaCl were obtained by 3-fold stepwise dilutions starting from 1 M. The mean ion activity, $a_{\pm}$, was computed from the concentration of $Na^+$ and $Cl^-$ at each pH using activity coefficients reported elsewhere (Lewis & Randall, 1961). Temperature effects were studied in the temperature range 10–35 °C, using the triple buffer in the presence of 0.1 M NaCl. 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 computed according to $\Delta pH/\Delta T$ coefficients reported elsewhere (Di Cera et al., 1991).

*Data Analysis.* The linkage expressions used in data analysis and the parameters involved have been derived and defined elsewhere (Di Cera et al., 1991) and are not duplicated here. Equations 19 and 20 of the paper by Di Cera et al. (1991) were used to analyze values of $K_m$ and $k_{cat}$ collected at different pHs as a function of NaCl concentration, while the complete data set of $K_m$ and $k_{cat}$ values obtained at different pHs and temperatures was globally analyzed using eq 21 and 22, with the definitions given in eq 23–25.

*Control Experiments.* Control experiments were run to check both reproducibility and accuracy of the results. Since the structural perturbation of human $\gamma$-thrombin is such that the enzyme is split into three different domains linked noncovalently (Villanueva, 1981; Berliner, 1984), it is absolutely necessary to establish that steady-state parameters are not affected by $\gamma$-thrombin dissociation at low concentrations. This important aspect, that is of course irrelevant in the case of the native enzyme, cannot be overlooked in quantitative thermodynamic studies of the functional properties of human $\gamma$-thrombin. Steady-state kinetics of substrate hydrolysis were run as a function of human $\gamma$-thrombin concentration in the range 0.1–1 nM, and no significant difference was found in the values of $k_{cat}$ and $K_m$. This provides direct evidence that

[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; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-2238, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane.

Table I: Effect of [NaCl] on the Parameters Involved in Binding and Catalysis[a]

| | Effect of [NaCl] (M) on p$K$ Values of Ionizable Groups | | | | | |
|---|---|---|---|---|---|---|
| | free enzyme | | | adduct | | |
| [NaCl] | p$K_1$ | p$K_2$ | p$K_3$ | p$K_1$ | p$K_2$ | p$K_3$ |
| 1.000 | 6.69 ± 0.23 | 8.89 ± 0.15 | 7.15 ± 0.25 | 5.84 ± 0.21 | 9.64 ± 0.15 | 7.15 ± 0.25 |
| 0.333 | 6.91 ± 0.21 | 8.78 ± 0.13 | 7.38 ± 0.29 | 6.14 ± 0.29 | 9.53 ± 0.13 | 7.38 ± 0.29 |
| 0.111 | 6.86 ± 0.13 | 8.74 ± 0.10 | 7.73 ± 0.21 | 6.20 ± 0.22 | 9.40 ± 0.12 | 7.73 ± 0.21 |
| 0.037 | 7.47 ± 0.12 | 8.56 ± 0.12 | 7.67 ± 0.28 | 6.29 ± 0.18 | 9.83 ± 0.11 | 7.67 ± 0.28 |
| 0.012 | 7.61 ± 0.21 | 8.23 ± 0.16 | 7.54 ± 0.20 | 5.51 ± 0.35 | 9.85 ± 0.11 | 7.54 ± 0.20 |
| 0.004 | 7.49 ± 0.28 | 8.42 ± 0.22 | 7.34 ± 0.30 | 5.17 ± 0.31 | 9.99 ± 0.20 | 7.34 ± 0.30 |
| 0.001 | 7.73 ± 0.22 | 8.17 ± 0.21 | 7.49 ± 0.16 | 4.34 ± 0.43 | 9.96 ± 0.13 | 7.49 ± 0.22 |

| | Effect of [NaCl] (M) on Acylation Rates ($s^{-1}$) and Substrate Dissociation Constant $^0K_d$ ($\mu$M)[b] | | | | | |
|---|---|---|---|---|---|---|
| [NaCl] | $^0k_2$ | $^1k_2$ | $^2k_2$ | $^3k_2$ | $^0K_d$ | $\sigma$ |
| 1.000 | 25.8 ± 15.0 | 159.3 ± 92.6 | 45.9 ± 23.0 | 52.1 ± 20.8 | 16.2 ± 2.5 | 0.032 |
| 0.333 | 14.6 ± 12.4 | 153.4 ± 97.4 | 50.1 ± 21.0 | 47.2 ± 28.3 | 14.3 ± 2.0 | 0.036 |
| 0.111 | 18.0 ± 6.6 | 167.8 ± 67.1 | 48.4 ± 16.0 | 33.7 ± 16.2 | 18.0 ± 6.6 | 0.023 |
| 0.037 | 5.7 ± 1.4 | 93.4 ± 28.0 | 33.6 ± 10.1 | 16.7 ± 6.0 | 56.5 ± 13.7 | 0.047 |
| 0.012 | 5.3 ± 1.3 | 64.0 ± 16.0 | 11.6 ± 3.5 | 6.7 ± 4.0 | 53.1 ± 13.7 | 0.051 |
| 0.004 | 5.8 ± 0.8 | 54.2 ± 10.8 | 7.7 ± 3.1 | 25.0 ± 13.7 | 58.0 ± 7.7 | 0.061 |
| 0.001 | 10.4 ± 2.9 | 44.6 ± 11.6 | 8.5 ± 1.7 | 13.9 ± 8.4 | 104.4 ± 29.0 | 0.053 |

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

γ-thrombin does not dissociate significantly in the concentration range employed in our studies.

Our effects, such as the reversed linkage with NaCl at pH <7 (see Results), were checked by repeated measurements using different γ-thrombin preparations with different concentrations. The results reported in a previous study on the effect of NaCl on α-thrombin (Di Cera et al., 1991) were also checked in the same pH range with different thrombin preparations and found to be perfectly reproducible.

The hypothesis that $K_m$ corresponds to the dissociation constant for substrate binding to the enzyme was also carefully checked. In the case of α-thrombin, this hypothesis was proven to be correct by measurements of $K_m$ for a poor substrate for thrombin, BzArgNHNp, and of $K_I$ for the competitive inhibitor *p*-aminobenzamidine (Di Cera et al., 1991) carried out over the same pH range. In the case of γ-thrombin, only *p*-aminobenzamidine could be used. Due to the extremely low value of the ratio $k_{cat}/K_m$, a few control experiments with BzArgNHNp would have required 10 times more γ-thrombin than all experiments carried out in the present study with S-2238. The pH dependence of $K_m$ for S-2238 and $K_I$ for *p*-aminobenzamidine was found to be controlled by two ionizable groups with the same p$K$ values for the free enzyme, consistent with $K_m$ being the dissociation constant of S-2238 (Cleland, 1982; Di Cera et al., 1991).

## Results

The pH dependence of the Michaelis–Menten parameters $K_m$ and $k_{cat}$ for human γ-thrombin is shown in Figure 1, and the best-fit values of the parameters involved in the analytical expressions 4 and 5 are listed in Table I. As for human α-thrombin, the pH dependence of $K_m$ shows a bell-shaped curve. The pH dependence of $k_{cat}$ is also bell-shaped, with a maximum around pH 8.5. Among the three ionizable residues responsible for the pH dependence of the Michaelis–Menten parameters, two are involved in the control of $K_m$ (p$K_1$ and p$K_2$ in Table I) and change their p$K$ upon substrate binding to the enzyme. The third group (p$K_3$ in Table I) shows no significant change in p$K$ with substrate binding and hence controls $k_{cat}$ along with the other two groups. The p$K$ values obtained for γ-thrombin as a function of NaCl concentration reveal the extent of perturbation induced by the structural modification of α-thrombin. Comparison of the p$K$ values with those of α-thrombin (Di Cera et al., 1991) points out the perturbation of residues involved in the control of binding and

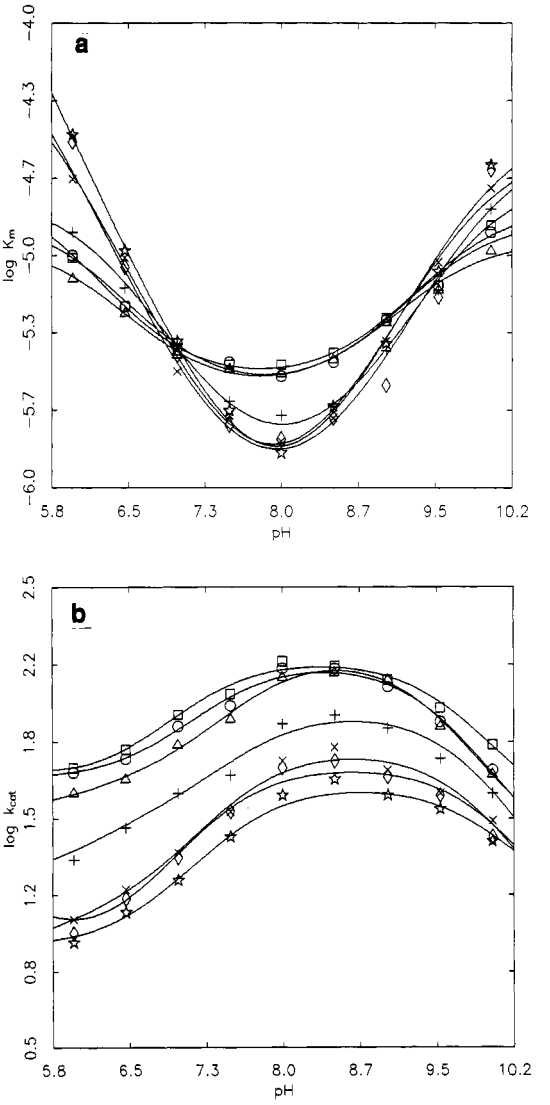


FIGURE 1: Results of the pH dependence of the Michaelis–Menten parameters $K_m$ (a) and $k_{cat}$ (b) for human γ-thrombin amidase activity as a function of [NaCl]. Points are best-fit values obtained from analysis of experimental data using eq 2. Continuous lines were drawn from eq 19–20 of the paper by Di Cera et al. (1991) using the parameter values listed in Table I. 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.

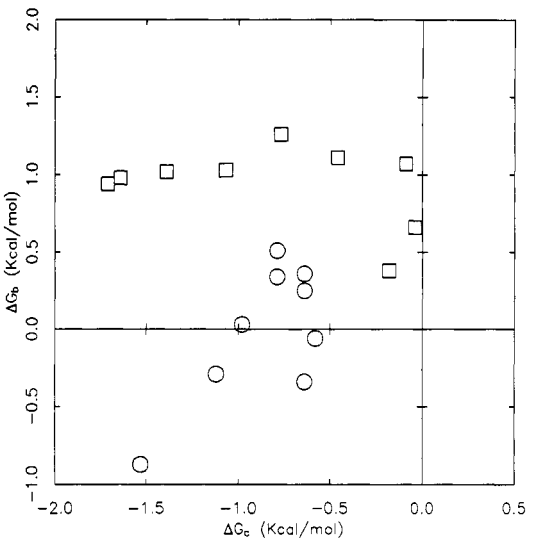


FIGURE 2: Linkage graph for the effect of NaCl concentration on thrombin amidase activity. Each point represents the value of $\Delta G_b$ as a function of $\Delta G_c$ (see eq 3) at different pHs (from 6 to 10, clockwise). The graph has been constructed with the data shown in Figure 1 for γ-thrombin (○) and data reported elsewhere (Di Cera et al., 1991) for the native enzyme (□). The drastic difference in the two patterns shows in a direct way the thermodynamic aspects of the structural perturbation of human γ-thrombin as compared to the native enzyme.

catalysis is evident. The first residue is mostly perturbed at low NaCl concentrations, most notably in the adduct. The second residue, on the other hand, is more affected at high NaCl concentrations in both the free enzyme and adduct. The third residue involved only in the control of $k_{cat}$ is significantly perturbed, from −0.27 to −1.22 p$K$ units, over the entire range of NaCl concentrations.

The perturbation of p$K$ values is such that the dependence of $K_m$ on [NaCl] shows two crossover points at pH 7 and 9.5 (see Figure 1a), which is a distinctive feature of γ-thrombin as compared to the native enzyme. In the case of α-thrombin, the linkage between [NaCl] and substrate binding is always negative; i.e., increasing NaCl concentration increases $K_m$ and hence decreases substrate affinity independent of pH (Di Cera et al., 1991). In the case of γ-thrombin, the presence of crossover points makes the linkage with [NaCl] more complex. The linkage is negative in the pH range 7.5–9, vanishes at the crossover points, and reverses itself outside this pH range. The overall energetics involved in this linkage are encapsulated by the free energy values:

$$\Delta G_b = RT\int_{-\infty}^{+\infty}(\partial \ln K_m/\partial \ln a_\pm)\, d \ln a_\pm = RT \ln [K_m(+\infty)/K_m(-\infty)] \quad (3a)$$

$$\Delta G_c = -RT\int_{-\infty}^{+\infty}(\partial \ln k_{cat}/\partial \ln a_\pm)\, d \ln a_\pm = -RT \ln [k_{cat}(+\infty)/k_{cat}(-\infty)] \quad (3b)$$

where $\Delta G_b$ is the free energy change for substrate binding observed when the mean ion activity of the salt, $a_\pm$, changes from 0 to infinity and likewise $\Delta G_c$ is the free energy change for catalysis. In the absence of linkage, both $\Delta G_b$ and $\Delta G_c$ are zero, while a positive value of $\Delta G_b$ or $\Delta G_c$ denotes negative linkage and vice versa. The relevant free energies of linkage can be derived from a plot of ln $K_m$ or ln $k_{cat}$ versus the logarithm of mean ion activity $a_\pm$ as the spacing between the asymptotes reached at very low and high values of $a_\pm$. The free energy values obtained in this way are independent of any particular mechanism that may be invoked to explain the

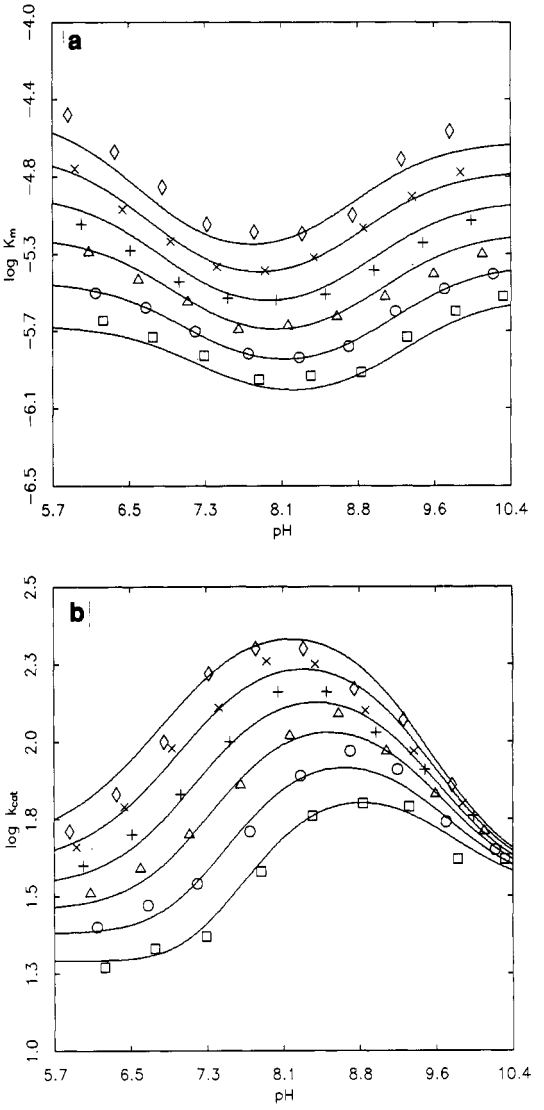


FIGURE 3: Results of the pH dependence of the Michaelis–Menten parameters $K_m$ (a) and $k_{cat}$ (b) for human α-thrombin amidase activity as a function of temperature. Points are best-fit values obtained from analysis of experimental data using eq 2. The pH values at all temperatures were calculated using $\Delta pH/\Delta T$ coefficients reported elsewhere (Di Cera et al., 1991). Continuous lines were drawn from eq 21–25 of the paper by Di Cera et al. (1991) using the parameter values listed in Table II. Temperature values are as follows: (□) 10 °C; (○) 15 °C; (△) 20 °C; (+) 25 °C; (×) 30 °C; (◇) 35 °C.

dependence of $K_m$ or $k_{cat}$ on salt concentration and are therefore useful phenomenological parameters that quantify the effect of NaCl, as well as any other linked effector, on the relevant parameters $K_m$ and $k_{cat}$ that reflect binding and catalytic events. A plot of $\Delta G_b$ versus $\Delta G_c$ is revealing of the thermodynamic basis for the linkage observed experimentally, independent of any mechanism, and is shown in Figure 2 for both γ-thrombin and the native enzyme. The figure shows the dramatic difference between the two enzymes in a rather straightforward way, with γ-thrombin undergoing larger changes in $\Delta G_b$ and smaller changes in $\Delta G_c$ as compared to α-thrombin as a function of pH. In particular, $\Delta G_b$ is practically pH-independent for the native enzyme from pH 6 to 9, while in the same pH range $\Delta G_b$ changes of about 1.5 kcal/mol are seen for γ-thrombin. On the other hand, the change in $\Delta G_c$ over the same pH range is about 0.8 kcal/mol smaller for γ-thrombin.

The effect of temperature on the pH dependence of human α-thrombin amidase activity is shown in Figure 3, and the

Table II: Best-Fit Values of the Parameters Involved in Binding and Catalysis and Corresponding van't Hoff Enthalpies and Activation Energies

| Thermodynamic Parameters for Substrate and Proton Binding[b] | | |
|---|---|---|
| free enzyme | ${}^0K_d{}^0 = 10.9 \pm 0.6$ | ${}^0\Delta H_s = -13.42 \pm 1.02$ |
| | $pK_1 = 7.18 \pm 0.26$ | ${}^E\Delta H_1 = 6.53 \pm 0.61$ |
| | $pK_2 = 8.64 \pm 0.23$ | ${}^E\Delta H_2 = 8.85 \pm 1.40$ |
| | $pK_3 = 7.48 \pm 0.36$ | ${}^E\Delta H_3 = 10.76 \pm 1.12$ |
| adduct | $pK_1 = 6.49 \pm 0.27$ | ${}^{ES}\Delta H_1 = 12.83 \pm 1.68$ |
| | $pK_2 = 9.29 \pm 0.20$ | ${}^{ES}\Delta H_2 = 7.52 \pm 1.49$ |
| | $pK_3 = 7.48 \pm 0.36$ | ${}^{ES}\Delta H_3 = 10.76 \pm 1.12$ |
| Thermodynamic Parameters for Catalysis[c] | | |
| ${}^0k_2{}^0 = 33.8 \pm 5.6$ | | $\Delta E_0 = 0.42 \pm 0.34$ |
| ${}^1k_2{}^0 = 159.2 \pm 31.8$ | | $\Delta E_1 = 8.01 \pm 1.28$ |
| ${}^2k_2{}^0 = 44.6 \pm 14.3$ | | $\Delta E_2 = 9.53 \pm 1.68$ |
| ${}^3k_2{}^0 = 33.8 \pm 6.1$ | | $\Delta E_3 = 5.54 \pm 1.59$ |

[a] Derived from global analysis of the data shown in Figure 3 according to eq 21–25 of the paper by Di Cera et al. (1991). Errors are at the cutoff of one standard deviation. [b] The substrate dissociation constant is in micromolar while standard enthalpy values are in kilocalories per mole. $pK$ values and ${}^0K_d{}^0$ are standard values calculated at 0.1 M NaCl, 0.1% PEG 8000, and 25 °C, under the buffer conditions given in the text. [c] The acylation rates are in $s^{-1}$ while energies of activation are in kilocalories per mole. $k_2{}^0$ values are standard values calculated at 0.1 M NaCl, 0.1% PEG 8000, and 25 °C, under the buffer conditions given in the text.

best-fit values of the parameters involved in the linkage scheme are listed in Table II. Equations 21–25 of the paper by Di Cera et al. (1991) give a very good fit of the data ($\sigma = 0.039$) and allow for resolution of all relevant enthalpic components to binding and catalytic events. The parameter values computed at the standard state (25 °C) are in 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 perturbed to different extents with respect to the native enzyme. In the free enzyme, it is particularly evident in the perturbation of the second ionizable group, which has a $\Delta H$ value of about 4.5 kcal/mol smaller than that found for the native enzyme, as well as by a difference of 1.8 kcal/mol in ${}^E\Delta H_3$. In the adduct, there is a notable change in the enthalpy of ionization of the first residue, which is over 5 kcal/mol more endothermic than in the native enzyme. All these changes bring about differences in the binding and catalytic properties of the enzyme as compared to those of α-thrombin. The relevant thermodynamic parameters have been derived from eq 26–28 of the paper by Di Cera et al. (1991) and are listed in Table III. The structural perturbation of γ-thrombin seems to affect mostly the enthalpy of substrate binding, especially in the end regions of the pH range explored, with a resulting increase in the negative entropic contribution. The energy of activation is also perturbed and is smaller than that of the native enzyme independent of pH. It should also be pointed out that the values of $\Delta H_s$ are independent of temperature at each pH, and so are the values of $\Delta E$. This observation unequivocally demonstrates that there is no change in the rate-limiting step over the pH range studied, so that acylation is slower than deacylation and substrate dissociation (Laidler, 1969; Laidler & Peterman, 1979), and the $K_m$ is equivalent to the substrate dissociation constant as in the case of human α-thrombin.

Table III: Thermodynamic Parameters for Substrate Binding and Catalysis as a Function of pH, under Experimental Conditions of 0.1 M NaCl, 0.1% PEG 8000, and 25 °C[a]

| pH | $\Delta G_s$ | $\Delta H_s$ | $T\Delta S_s$ | $\Delta E$ |
|---|---|---|---|---|
| 6.00 | −6.86 ± 0.21 | −16.00 ± 0.96 | −9.14 ± 0.98 | 8.08 ± 0.49 |
| 6.50 | −7.03 ± 0.21 | −13.67 ± 0.82 | −6.64 ± 0.85 | 10.29 ± 0.62 |
| 7.00 | −7.27 ± 0.22 | −11.84 ± 0.71 | −4.56 ± 0.74 | 12.41 ± 0.74 |
| 7.50 | −7.46 ± 0.22 | −11.74 ± 0.70 | −4.28 ± 0.74 | 12.36 ± 0.74 |
| 8.00 | −7.51 ± 0.23 | −12.82 ± 0.77 | −5.31 ± 0.80 | 10.45 ± 0.63 |
| 8.50 | −7.41 ± 0.22 | −14.38 ± 0.86 | −6.96 ± 0.89 | 8.41 ± 0.50 |
| 9.00 | −7.20 ± 0.22 | −15.51 ± 0.93 | −8.30 ± 0.96 | 6.33 ± 0.38 |
| 9.50 | −6.99 ± 0.21 | −15.25 ± 0.91 | −8.26 ± 0.94 | 3.98 ± 0.24 |
| 10.00 | −6.86 ± 0.21 | −14.36 ± 0.86 | −7.50 ± 0.89 | 2.06 ± 0.12 |

[a] All values are given in kilocalories per mole, and errors are at the cutoff of one standard deviation.

## Discussion

Previous studies on human γ-thrombin have pointed out some basic differences with respect to the native enzyme, namely, the fact that γ-thrombin does not clot fibrinogen, binds hirudin with lower affinity, and is less stable than α-thrombin (Lundblad et al., 1979; Villanueva, 1981; Fenton, 1986; Stone & Hofsteenge, 1991). The results reported in this study confirm that γ-thrombin is less stable than α-thrombin since steady-state data could only be collected over a narrower temperature range, namely, from 10 to 35 °C. The thermodynamic aspects of binding and catalytic events for the structurally perturbed enzyme also show significant differences in their enthalpic and entropic components. The energy of activation is decreased and substrate binding is significantly more exothermic, especially at low and high pH values, with a concomitant increase in the negative entropic changes. The free energy of substrate binding, on the other hand, is not significantly affected. The information collected in this study is useful to characterize the energetic aspects of the structural perturbation of γ-thrombin, which still lacks a quantitative characterization in terms of X-ray crystallography. Both binding and catalytic properties of the enzyme are affected by the structural perturbation, although to different extents depending upon solution conditions. Under experimental conditions of 0.1 M NaCl, the values of $\Delta G_s$ for S-2238 are only slightly different from those of the native enzyme over the entire pH range examined in this study, but the enthalpy and entropy of binding are very different and so is the activation energy. A common trend is the variation of $\Delta H$ values for binding and catalysis indicates the presence of more exothermic processes. These processes seem to be compensated by increased entropy changes in the case of binding events, so that the free energy of substrate binding is only slightly affected. Upon substrate binding, the entropy decreases more in γ-thrombin than in the native enzyme. Since the free energy of binding is almost the same for both enzymes, one may speculate that substrate binding stabilizes the free form of γ-thrombin and keeps it in a conformation similar to that of the native enzyme. Consequently, free γ-thrombin would be a more "disordered" enzyme than α-thrombin, which is consistent with the fact that the digestion with trypsin generates three noncovalently linked structural domains from a single polypeptide chain with a resulting less compact structure than the native enzyme (Villanueva, 1981). More extensive structural rearrangements occurring upon substrate binding would also give rise to different enthalpy changes. Differential hydration and hydrophobic effects along with perturbation of ionization reactions may account for the more exothermic trend observed experimentally.

The structural perturbation in γ-thrombin must be such to affect at least one of the three ionizable residues, namely, the group that controls $k_{cat}$ but not $K_m$, in a significant and direct way. Our recent suggestion that this group may be located in the ABE (Di Cera et al., 1991), which is drastically perturbed in human γ-thrombin, seems to be supported by the present findings although alternative possibilities cannot be

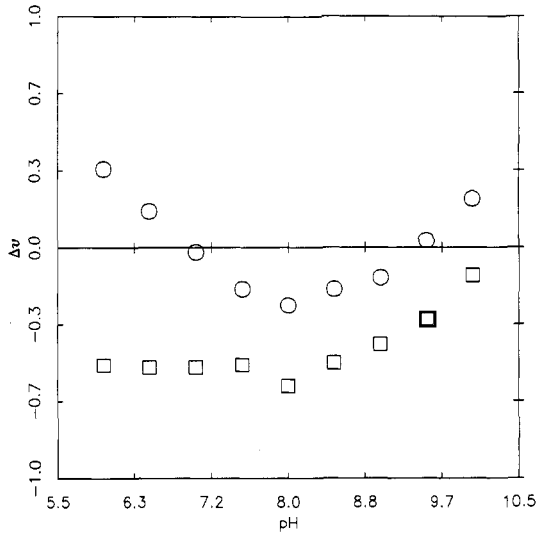


FIGURE 4: Linkage graph for the effect of NaCl concentration on thrombin amidase activity. Each point represents the maximum number of ions (cations and anions) released ($\Delta\nu < 0$) or uptaken ($\Delta\nu > 0$) upon substrate binding to the enzyme, as a function of pH. The figure shows the difference between γ-thrombin (○) and the native enzyme (□), for which the linkage with NaCl is always negative (Di Cera et al., 1991). The reduced absolute value of $\Delta\nu$ observed in the case of γ-thrombin strongly supports the idea that the structural perturbation affecting the ABE may prevent chloride binding to this site.

ruled out at this stage. The p$K$ values of the other two ionizable groups controlling both $K_m$ and $k_{cat}$ are affected at very high or low NaCl concentrations, while shows only a slight change around 0.1 M NaCl. However, the enthalpy of ionization of these residues is drastically perturbed, in both the free enzyme and adduct, with the exception of $^{E}\Delta H_1$ (see Table II). This suggests an indirect but significant perturbation of the ionization reactions linked to the structural perturbation of human γ-thrombin, probably mediated by differential hydration effects and structural rearrangements linked to the protonation/deprotonation of the active-site His and the amino terminus of the B chain.

Perhaps the most unexpected difference between γ-thrombin and the native enzyme is the one found in the dependence of $K_m$ on NaCl concentration. We have already shown that human α-thrombin is capable of discriminating among different salts and that the effect of NaCl on thrombin amidase activity is probably mediated by specific ion binding interactions (Di Cera et al., 1991). If this is indeed the case, then the ABE may play a critical role in the modulation of thrombin activity by salts by providing a binding site for small anions. Recent structural studies have pointed out a unique spatial arrangement of three basic residues of the ABE, Arg67, Arg75, and Arg77A (Rydel et al., 1990), that form a triangle of positive charges that seem to be close enough to provide a binding site for small anions. One of the cleavages by trypsin to yield γ-thrombin occurs at Arg73, just two residues away from Arg75 in the putative binding site for small anions. The reversed linkage observed with human γ-thrombin at pH <7 and >9.5 may well be due to the perturbation of this triangle of Arg residues. A particularly attractive hypothesis is that thrombin amidase activity may be controlled by several ion binding sites, one of them being the triangle of Arg residues in the ABE. This hypothesis seems to be supported by the observation that the salt dependence of $K_m$ and $k_{cat}$ for human γ-thrombin and the native enzyme is such to suggest involvement of at least two ion binding sites. The structural perturbation of human γ-thrombin would prevent ion binding to the ABE, so that the salt dependence of $K_m$ and $k_{cat}$ for this derivative would strictly reflect the linkage between the catalytic pocket and the remaining ion binding sites other than the ABE. This is consistent with experimental data of the salt dependence of $K_m$ for γ-thrombin reported here. Under conditions where aspecific ionic strength effects seem to play only a secondary role, as in the case of human thrombin (Di Cera et al., 1991), the slope $\Delta\nu = -\mathrm{d}\ln K_m/\mathrm{d}\ln a_{\pm}$ is a direct measure of the net number of ions released ($\Delta\nu < 0$) or taken up ($\Delta\nu > 0$) upon substrate binding to the enzyme (Record et al., 1978). This quantity is plotted in Figure 4 for both γ-thrombin and the native enzyme and shows quite nicely the thermodynamic bases of the reversed linkage observed in the case of γ-thrombin. The reduced (absolute) value of $\Delta\nu$ observed in the case of γ-thrombin as compared to the native enzyme is consistent with the hypothesis that the structural perturbation has reduced the number of ion binding sites available. The reversed linkage would therefore be a consequence of the binding properties of fewer sites that are decoupled from the structurally perturbed ABE. In thermodynamic terms, the linkage between ion binding and amidase activity is *global* in the case of the native enzyme since it involves all ion binding sites and is *local* in the case of the structurally perturbed derivative since it involves a reduced number of specific binding sites. The significant difference in ion binding between the native and structurally perturbed enzyme would thus be a consequence of the difference observed in the global and local description of ligand binding to a biological macromolecule (Di Cera, 1990). Human γ-thrombin may act as a "contracted form" of the native enzyme where the ABE is constrained in its unligated form by the structural perturbation.

The foregoing hypothesis brings together functional and structural findings in a consistent picture. The information on the properties of the enzyme collected here and in a previous study (Di Cera et al., 1991) seems to indicate that it would be possible for the thrombin system to derive site-specific, ion binding energetics from structural perturbations, as predicted by the theory of "contracted partition functions" (Di Cera, 1990). As further information is gathered on the behavior of the thrombin system in the presence of different salts (other than NaCl), detailed partition functions for ion binding can be constructed along with their "contracted" forms. This will allow for a complete thermodynamic dissection of the ion binding properties of the enzyme that seem to be of relevance for the modulation of its activity in vivo, as indicated by recent findings on thrombin–fibrinogen interaction (De Cristofaro & Di Cera, 1992).

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# Identification of Two Cysteine Residues Forming a Pair of Vicinal Thiols in Glucosamine-6-phosphate Deaminase from *Escherichia coli* and a Study of Their Functional Role by Site-Directed Mutagenesis†

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ABSTRACT: The nucleotide sequence of the *nagB* gene in *Escherichia coli*, encoding glucosamine-6-phosphate deaminase, located four cysteinyl residues at positions 118, 219, 228, and 239. Chemical modification studies performed with the purified enzyme had shown that the sulfhydryl groups of two of these residues form a vicinal pair in the enzyme and are easily modified by thiol reagents. The allosteric transition to the more active conformer (R), produced by the binding of homotropic (D-glucosamine 6-phosphate or 2-deoxy-2-amino-D-glucitol 6-phosphate) or heterotropic (*N*-acetyl-D-glucosamine 6-phosphate) ligands, completely protected these thiols against chemical modification. Selective cyanylation of the vicinal thiols with 2-nitro-5-(thiocyanato)benzoate, followed by alkaline hydrolysis to produce chain cleavage at the modified cysteines, gave a pattern of polypeptides which allowed us to identify Cys118 and Cys239 as the residues forming the thiol pair. Subsequently, three mutated forms of the gene were constructed by oligonucleotide-directed mutagenesis, in which one or both of the cysteine codons were changed to serine. The mutant proteins were overexpressed and purified, and their kinetics were studied. The dithiol formed by Cys118 and Cys239 was necessary for maximum catalytic activity. The single replacements and the double mutation affected catalytic efficiency in a similar way, which was also identical to the effect of the chemical block of the thiol pair. However, only one of these cysteinyl residues, Cys239, had a significant role in the allosteric transition, and its substitution for serine reduced the allosteric interaction energy, due to a lower value of $K_T$.

Glucosamine-6-phosphate deaminase catalyzes the reversible conversion of D-glucosamine 6-phosphate (GlcN6P)[1] into D-fructose 6-phosphate and ammonia. It is allosterically activated by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P) (Comb & Roseman, 1958; Midelfort & Rose, 1977; Calcagno et al., 1984). The enzyme from *Escherichia coli* is an oligomeric protein composed of six identical polypeptide chains (Calcagno et al., 1984) whose primary structure is known from the DNA sequence of the gene *nagB* encoding this enzyme (Rogers et al., 1988). The gene, located at 15.5 min on the *E. coli* chromosome (White, 1968; Holmes & Russell, 1972),

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; NTCB, 2-nitro-5-(thiocyanato)benzoic acid; SDS, sodium dodecyl sulfate; TNB, 2-nitro-5-thiobenzoate.

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