The non-Michaelian action of thrombin on peptide p-nitroanilide substrates

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INTRODUCTION
Peptide p-nitroanilide substrates for thrombin and other coagulation proteinases permit the introduction of a new methodology in the field of clinical analysis based on the hydrolysis of these substrates. For thrombin, interest has mainly focused on obtaining increasingly specific p-nitroanilide peptides and on analysing their clinical applications. By contrast, the kinetic aspects of their hydrolysis have not received much attention.

Data regarding native bovine thrombin may be found in the contributions to this field by Svendsen et al. (1972), Hijikata et al. (1979) and Soria et al. (1980), who determined the kinetic parameters $K_m$ and $V_{max}$ (or $k_{cat}$) for different synthetic peptides of p-nitroanilide by using Lineweaver–Burk plots. Pozsgay et al. (1981) and Svendsen & Stocker (1977) performed similar determinations in the case of native human thrombin; in a review, Lottenberg et al. (1981) offered kinetic data concerning both native human and bovine thrombin against different peptide p-nitroanilide substrates. They reported that the $v$/[S] data fit the Michaelis–Menten equation, though they also point to evidence of “activation by substrate”.

Taking into account that both in the case of human and bovine thrombin there is clear evidence demonstrating the existence of at least three isoenzymes, known as $\alpha$, $\beta$ and $\gamma$-thrombin (Lundblad et al., 1977; Fenton et al., 1979), some investigators have studied these enzymes in isolation. These studies gave $K_m$ and $V_{max}$ values from Lineweaver–Burk plots for bovine $\alpha$-thrombin against substrate S-2238 (Okamoto et al., 1981) and those corresponding to $\alpha$- and bovine $\beta/\gamma$-thrombin against Tos-Ch-TH and S-2238 (Strukova et al., 1980). Likewise, Orthner & Kosow (1980) determined kinetic parameters for human $\alpha$-thrombin against substrates S-2238, and Griffith et al. (1980) also determined them against Tos-Ch-TH, although on that occasion using the integrated Michaelis–Menten equation.

The recent works of Lottenberg’s group are of special interest. Lottenberg et al. (1982) studied the hydrolysis of the substrates Tos-Ch-TH and S-2238 both by human and bovine $\alpha$-thrombin and by the degraded forms of human $\alpha$-thrombin and bovine $\beta$-thrombin. A Michaelian kinetic behaviour of the enzyme towards these substrates was assumed, and as proof of this, they mentioned the fact that the plotting of the residuals against the substrate concentration seemed to follow a random distribution. This condition is necessary, but not sufficient, to conclude goodness-of-fit. The same workers also pointed out that, owing to the low thrombin concentration required for the hydrolysis of these substrates, the greatest sources of error in the determinations were derived from losses of enzymic activity by adsorption, and their work focused on the determination and comparison of the kinetic parameters $K_m$ and $k_{cat}$ for the different forms of human and bovine thrombin. More recently (Lottenberg et al., 1983) the group has broadened the scope of its research, analysing with the same methodology the hydrolysis of 24 commercial peptide p-nitroanilides by bovine $\alpha$-thrombin under different experimental conditions. Hence, up to the present, all the works published share a common feature: the assumption that the Michaelis–Menten rate equation is the most suitable to describe the kinetic behaviour of thrombin in the hydrolysis of different chromogenic substrates. However, is it really true that thrombin exhibits Michaelis–Menten behaviour in these reactions?

Abbreviations used: S-2238, H-D-Phe-L-Pip-L-Arg-pNA (where Pip is piperidyl and pNA is p-nitroanilide); S-2288, H-D-Ile-L-Pro-l-Arg-pNA; Tos-Ch-TH, Tos-Gly-L-Pro-l-Arg-pNA (where Tos is tosyl); Cbz-Ch-TH, Cbz-Gly-L-Pro-l-Arg-pNA (where Cbz is benzyloxy carbonyl).
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The studies published up to now do not seem to have considered this aspect in depth. Our own experience in the treatment of kinetic data (Bur guillo et al., 1983; Del Arco et al., 1985) has provided us with a suitable method for the establishment, by statistical means, of the minimum degree of the rate equation of an enzyme. To do so, numerous \( v(S) \) kinetic data were obtained, each experimental point being determined as the mean of five replicate measurements. These kinetic sets were then fitted by non-linear regression methods to rate equations of the increasing-degree polynomial quotient type, discriminating between equations of different degree by applying the \( F \)-test to the respective sum of weighted squared residuals.

Accordingly, the aim of the present work was to establish, by using this methodology, the minimum degree of the rate equation for the hydrolysis of different chromogenic substrates by native human thrombin.

**MATERIALS AND METHODS**

**Reagents**

Thrombin (EC 3.4.21.5) from human plasma was supplied by Sigma in a freeze-dried form (T-6759). The commercial preparation exhibited a minimum specific activity of 3000 NIH (National Institutes of Health) units/mg of protein and was considered to be free of other coagulation factors, plasminogen and plasmin. SDS/polyacrylamide-gel-electrophoretic analysis showed the existence of two major protein bands with \( M_r \) values close to 36000 and 28000 as measured with a reference kit. According to the data found in the literature (Lundblad et al., 1977; Fenton et al., 1979), these two bands appeared to correspond to \( \alpha \) and \( \beta \)-thrombin isoenzymes. A third minor band was also observed with an \( M_r \) below 14000, possibly due to some impurity present in the preparation.

The freeze-dried thrombin was reconstituted in 0.05 m-citrate/0.15 m-NaCl, pH 7.2, in order to obtain concentrations of the order of 10 NIH units/ml and was stored in portions at a temperature below 0 °C. Working solutions were prepared immediately before each experiment by thawing a portion and suitably diluting it with 0.6 m-Tris/HCl buffer, pH 8.5 and \( I = 0.28 \), down to concentrations of 1 NIH unit/ml in polyethylene tubes. After this, 0.1% poly(ethylene glycol) 6000 was added to this solution. As suggested by Lottenberg et al. (1981, 1983) and confirmed by us, this agent is able to prevent losses in activity due to adsorption processes. Under the storage conditions described it was ascertained that, at temperatures between 0 and 5 °C, no de-activation of the enzyme took place during the customary working periods (5–8 h).

The chromogenic substrates S-2238 and S-2288 were a gift from Kabi Diagnostica in freeze-dried form, and the substrate CBz-Ch-Th was supplied in the freeze-dried acetate form by Boehringer-Mannheim. The substrate Tos-Ch-Th was supplied in acetate form, together with the other general reagents for high-purity analysis, by Sigma.

Substrate solutions were obtained by dissolving the corresponding freeze-dried material in distilled water and the real concentration of hydrolysable material was determined from the concentration of \( p \)-nitroaniline released by the action of thrombin at infinite time (\( e_{405} = 1.02 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1} \)). Working solutions were prepared immediately before each study by dilution of the stock solution in distilled water or the necessary buffer; no spontaneous hydrolysis of the substrates could be detected in a temperature range between 0° and 5 °C during the customary working periods.

**Kinetic procedures**

The kinetic studies carried out were based on a direct and continuous spectrophotometric measurement at 405 nm of \( p \)-nitroaniline released as a result of the hydrolysis of the different substrates by the action of thrombin.

The kinetic method chosen was the one involving initial rates, determining such rates from the slope at the origin of the absorbance–time curves. The calculation of this slope was performed numerically with a least-squares method and by considering hydrolysis periods in which the variation of absorbance with time was essentially linear; taking into account the molar absorption coefficient of \( p \)-nitroanilide at 405 nm, the rate was expressed in \( \mu \text{mol} \cdot \text{s}^{-1} \cdot \text{l}^{-1} \).

In order to obtain the absorbance–time kinetic curves over a wide range of substrate concentration, a high-sensitivity spectrophotometer was needed. Accordingly a Beckman DU-7 stabilized beam spectrophotometer with a controlling microcomputer and equipped with thermostatically controlled cuvettes was employed.

**Curve-fitting of experimental data**

We assumed a steady state with product concentration zero and an absence of significant enzyme association–dissociation ([E] in our experiments was always within the linear stretch of the \( v \) versus [E] plot (Izquierdo et al., 1985)). Hence, the steady-state equation is of the form (Wong, 1975):

\[
\frac{v}{[E]_0} = \frac{\alpha_1[S] + \alpha_2[S]^2 + \ldots + \alpha_n[S]^n}{\beta_0 + \beta_1[S] + \beta_2[S]^2 + \ldots + \beta_n[S]^n} = \frac{a_1[S] + a_2[S]^2 + \ldots + a_n[S]^n}{1 + b_1[S] + b_2[S]^2 + \ldots + b_n[S]^n}
\]

Curve-fitting and statistical analysis of data was performed on the Manchester CYBER 170-730 computer by using interactive programs incorporating subroutines from the NAG (Numerical Algorithms Group) Library, Oxford, U.K. Data were scaled to order unity, and initial slopes and asymptotes were estimated by linear regression to the first three and last three data points. Then a Monte Carlo search of parameter space was performed. The overdetermined linear equations were solved in the constrained \( L_1 \) norm, and the best set of initial parameter estimates was used in a constrained quasi Newton routine. The program performed regression to positive rational functions of order 1:1, 2:2, 3:3, \( F \)-tests for significant improvement in fit at each stage being performed.

Rate was expressed as \( \mu \text{mol} \cdot \text{s}^{-1} \cdot \text{l}^{-1} \) and substrate concentration in mol \( \cdot \text{l}^{-1} \). Curve-fitting was then carried out iteratively until the sum of squares was minimized. In some crucial sets, we measured the variance (\( \sigma^2 \)) of each rate from five replicate consecutive experiments and in those cases weighted regression analysis was used:

\[
Q_j = \sum_{i=1}^n w_i (v_i - f(\theta_i[S_i]))^2
\]
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Fig. 1. Eadie–Hofstee plots for the hydrolysis of peptide η-nitroanilide substrates
(a) S-2238; (b) S-2288; (c) Tos-Ch-TH; (d) Cbz-Ch-TH. Reaction conditions were: Tris/HCl, 0.11–0.13 M; poly(ethylene glycol) 6000, 0.1%; pH 8.1–8.5; I 0.17; temperature, 25–28 °C; thrombin concentrations ranged between 0.04 and 0.13 NIH unit/ml. ---, 1:1; ---, 2:2; ---, 3:3.

Table 1. Values of the sums of squared residuals (Q) obtained in the numerical fittings of r([S]) kinetic data to rational functions of degree n:n for the hydrolysis of chromogenic substrates by thrombin and the results of the F-test

Abbreviations: S99, significant at confidence levels of 99%; NS, not significant.

<table>
<thead>
<tr>
<th>Degree (n:n)</th>
<th>Substrate...</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-2238</td>
<td>S-2288</td>
</tr>
<tr>
<td>1:1</td>
<td>4.2298 × 10^1</td>
<td>3.9849 × 10^1</td>
</tr>
<tr>
<td>2:2</td>
<td>1.203</td>
<td>4.3422</td>
</tr>
<tr>
<td>3:3</td>
<td>2.6558 × 10^-1</td>
<td>9.7471 × 10^-1</td>
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</table>

F-test result

<table>
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<th>Comparison</th>
<th>Substrate...</th>
<th>S-2238</th>
<th>S-2288</th>
<th>Tos-Ch-TH</th>
<th>Cbz-Ch-TH</th>
</tr>
</thead>
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<tr>
<td>2:2 versus 1:1</td>
<td>S99</td>
<td>S99</td>
<td>NS</td>
<td>S99</td>
<td></td>
</tr>
<tr>
<td>3:3 versus 2:2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

where \( Q_j \) represents the weighted sum of squared residuals, \( w_i \) equals \( 1/\sigma_i^2 \), \( v_j \) refers to the initial rate and \( f \) is the rational function with \( \theta \) parameters.

Nevertheless, over the range of substrate concentrations employed, variance was approximately constant, so we designed the rest of the experiments without replicates and in these sets unweighted regression analysis was used.

Calculation of the F statistics
To discriminate between fittings to equations of successive degree and hence to be able to determine
degree \( n \) of the rate equation from which an increase in degree does not significantly improve the sum of squared residuals, the \( F \)-test was applied according to the formula proposed by Lindgren (1976):\[ F = \frac{(Q_1 - Q_2)/(m_2 - m_1)}{Q_2/(n - m_2)} \]

where \( Q_1 \) and \( Q_2 \) represent the sum of squared residuals for models 1 and 2, \( m_1 \) and \( m_2 \) refer to the corresponding number of parameters, and \( n \) refers to the number of experimental results. The value of \( F \) obtained in this way was referred to the corresponding confidence levels of 95 and 99%.

**RESULTS**

**Hydrolysis of peptide \( p \)-nitroanilide substrates by native human thrombin**

First, the variation of the hydrolysis rate with substrate concentration was analysed under conditions which, according to our previous results (Izquierdo et al., 1985, 1987) could be considered as optimum for hydrolysis of each of the substrates. Substrate concentration ranged between \( 2 \times 10^{-6} \) and \( 2 \times 10^{-4} \) M, and the rate

![Fig. 2. Eadie–Hofstee plots for the hydrolysis of peptide \( p \)-nitroanilide substrates obtained under very different experimental conditions](image)

(a) S-2238; (b) S-2288; (c) Tos-Ch-TH; (d) Cbz-Ch-TH. Reaction conditions were: Tris/HCl, 0.13–0.16 M; polyethylene glycol 6000, 0.1%; pH 8.4–8.6, \( I \) 0.17–0.18. Other conditions: (a) temperature 37.0 °C and 0.089 NIH unit of enzyme/ml; (b) temperature 15.0 °C and 0.13 NIH unit of enzyme/ml; (c) temperature 15.0 °C and 0.081 NIH unit of enzyme/ml; (d) temperature 15.0 °C and 0.089 NIH unit of enzyme/ml. ----: 1; , 2: 2; *- -*, 3: 3.

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**Table 2. Hydrolysis of chromogenic substrates by thrombin: summary of the results of the \( F \)-test for a broad range of experimental conditions (different temperatures, pH, ionic strength etc.)**

Note: success in detecting 3:3 > 2:2 with the \( F \)-test was never found. Curve fitting was carried out iteratively to minimize \( Q \), the weighted sum of squares. A full table with details about experimental conditions and \( Q \) values has been omitted to save space, but it may be obtained from the authors on request.

<table>
<thead>
<tr>
<th>Total number of curves fitted</th>
<th>Number of successes in detecting 2:2 &gt; 1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>At the 95% level</td>
<td>At the 99% level</td>
</tr>
<tr>
<td>24</td>
<td>17</td>
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</table>

in each case was obtained as the mean of five consecutive replicates, which yielded an estimation of the S.D. corresponding to each experimental point. Fig. 1 shows an Eadie–Hofstee plot of the experimental data and their
s.d. values and the theoretical curves corresponding to the fitting of degree 1:1, 2:2 and 3:3. In all cases it is possible to observe a deviation from linearity predicted by the Michaelis–Menten model (degree 1:1) and the improvement provided by the fitting of the experimental data to equations of higher degree (2:2 and 3:3). Numerically, a progressive decrease could be seen in the sum of squared residuals (Q) with the increase in the degree of the equation, and the results of the F-test showed that degree 2:2 significantly improved on degree 1:1, whereas no significant improvement was produced with degree 3:3 with respect to 2:2 (Table 1).

Similar several v([S]) studies were performed under other experimental conditions. Fig. 2 shows, as an example, some the curves obtained and, as may be seen, deviations from linearity can again be observed. The set of results obtained in our laboratory concerning the kinetic behaviour of native human thrombin towards chromogenic substrates is summarized in Table 2, whence it may be deduced that, for the total of 24 fitted curves distributed over a broad range of experimental conditions, in 71% of the cases the F-test detected a significant improvement of degree 2:2 with respect to degree 1:1, whereas in no case was such an improvement produced with degree 3:3 with respect to degree 2:2. Moreover, no important differences could be seen in the behaviour with the different substrates studied.

DISCUSSION

These results allow us to conclude that native human thrombin is an enzyme which shows non-Michaelian kinetic behaviour when hydrolysing chromogenic substrates and that we should consider the ‘minimum’ rate equation to be of degree 2:2, i.e.:

\[
\frac{v}{[E]_0} = \frac{a_2[S] + a_4[S]^2}{1 + b_1[S] + b_2[S]^2}
\]

Emphasis should be placed on the fact that this ‘minimum degree’ of 2:2 does not necessarily imply that this is the true degree of the reaction mechanism, though it might indeed coincide with it; neither does this exclude the possibility that, under certain experimental conditions, the particular values of the parameters \(a_2\) and \(b_1\) may lead to a masking of the degree, showing a behaviour of degree 1:1 (Burguillo et al., 1983). It appears, then, that our data do not rule out mechanisms of higher degree than the minimum one which perhaps are not manifest as such under experimental conditions but which do allow one to eliminate all those reaction mechanisms whose rate equations are lower than the minimum one.

Since electrophoretic analysis of our preparation of thrombin showed the presence of the \(\alpha\) and \(\beta\) isoenzymes of thrombin, there was a possibility that if both isoenzymes had different activity, our observations could be the result of the overlap of two Michaelian behaviours (rate equation of degree 1:1), which indeed would be manifested as an equation of degree 2:2. However, this equation would exhibit the peculiarity of having a denominator whose discriminant \((b_1^2 - 4b_2)\) must be, as may be seen, necessarily positive. Taking this into account we analysed this possible justification for our results on the basis of the values of the parameters \(b_1\) obtained in the fittings of degree 2:2 for all the studies carried out, with the finding that, in 75% of the cases, the discriminant was negative, which ruled out the possibility that we were in fact dealing with two Michaelian enzymes with different, but overlapping, activities. A stronger justification for excluding the high-order kinetics being explained by a mixture of two 1:1 isoenzymes follows from analysing the curvature of the data in Eadie–Hofstee space. A mixture of 1:1 isoenzymes gives a conic section which is uniformly concave up (Childs & Bardsley, 1976; Bardsley & Wood, 1985). However, most of the data is of the opposite type, i.e. uniformly concave-down, as will be clear from the Figures. Hence the curvature seems to be due to true kinetic complexity of the mechanism for one or both of the isoenzymes.

In view of these proofs, it would seem more logical to believe that \(\alpha\)- and \(\beta\)-thrombin hydrolyse these chromogenic substrates as two isoenzymes with the same activity and both of them with a more complex reaction mechanism than that of Michaelis–Menten, at least a mechanism of degree 2:2; this would account for the results observed in the present study.

Hence which model would be suitable for thrombin on p-nitroanilide substrates? If we consider the characteristics of the active site of thrombin, which shows different regions of secondary binding to which the substrate could have access (Fenton, 1981), a valid model could be the homosubstrate modifier mechanism (Scheme 1), where one considers the possible non-effective binding of a substrate molecule through one of the secondary binding sites without this preventing access of the substrate to the catalytic site itself, but which would indeed affect the breakage rate to give products. The rate equation corresponding to the mechanism shown in Scheme 1, according to the rules of King & Altman, is 3:3. Although this degree is higher than that detected experimentally in our studies (2:2), this in no way excludes such a mechanism, since the degree determined empirically, as mentioned above, is the ‘minimum degree’ and not the true degree of the reaction mechanism, which

Scheme 1. Homosubstrate modifier mechanism suggested for the action of native human thrombin on chromogenic substrates

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cannot be manifested mathematically at a sufficient level to prove experimentally detectable in a significant way (Burguillo et al., 1983).

In essence, a normal preparation of human thrombin (mainly composed of $\alpha$- and $\beta$-thrombin) exhibits non-Michaelian behaviour when hydrolysing chromogenic substrates; this could be of interest when one considers the catalytic properties of this enzyme and, furthermore, confirms our belief that a Michaelis–Menten behaviour for an enzyme is more the exception than the rule (Hill et al., 1977).

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REFERENCES


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