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LOCATING THE RATE-DETERMINING STEP(S) FOR THREE-STEP HYDROLASE-CATALYZED REACTIONS WITH DYNAFIT

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Summary

Hydrolytic reactions of oligopeptide 4-nitroanilides catalyzed by human- α -thrombin, human activated protein C and human factor Xa were studied at pH 8.0–8.4 and 25.0 ± 0.1 °C by the progress curve method and individual rate constants were calculated mostly within 10% internal error using DYNAFITV. A systematic strategy has been developed for fitting a three-step consecutive mechanism to eighteen hundred to six thousand time-course data points polled from two to four independent kinetic experiments. Enzyme and substrate concentrations were also calculated. Individual rate constants well reproduce published values obtained under comparable conditions and the Michaelis-Menten kinetic parameters calculated from these elementary rate constants are also within reasonable limits of published values. For comparison, the integrated Michaelis-Menten equation was also fitted to data from twelve sets. Both the k_{cat} and k_{cat}/K_m values are within 15% agreement with those calculated using the elementary rate constants obtained with DYNAFITV. Rate constants for the second and third consecutive steps are within 3–4 fold indicating that both determine the overall rate. The Factor Xa-catalyzed hydrolysis of N α -Z-D-Arg-Gly-Arg-pNA·2HCl at pH 8.4 in a series of buffers containing increasing fractions of deuterium at 25.0 ± 0.1 °C shows a very strong dependence of k_3 and a moderate dependence of k_2 on D content in the buffer: the fractionation factors are: 0.49 ± 0.03 for K_1 , 0.70 ± 0.05 for k_2 , and $(0.32 \pm 0.03)^2$ for k_3 .

Keywords

Enzyme kinetics; progress curve; elementary rate constants; blood cascade enzymes; solvent isotope effects; proton inventories

Introduction

Enzyme kinetic studies in the last decades have increasingly taken advantage of the revolution in computations. Software packages empower the user to fit a set of equations describing a reaction mechanism of multiple steps to a very large number of data points, even from a single time-dependent change in concentration. One of the most advanced and well-tested software for a range of enzyme kinetic calculations is DYNAFIT[1], incorporating the pioneering work of Garfinkel[2] and the experiences from programs KINSIM[3] and FITSIM.[4] These programs accept a given reaction scheme with elementary steps as input, and solve the requisite

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differential equations, *without specific assumptions*, by numerical integration. Two of the four novel features of DYNAFIT over its predecessors are that 1) it accepts initial concentrations of enzyme and substrate as fitting parameters after an initial guess, and 2) it can also treat as adjustable parameters molar absorptivity and baseline signal.

The two major gains of using enzyme kinetic software are: 1) the opportunity to account for increasingly complex multiple-step reactions at the level of elementary steps because all information is obtained from the entire reaction carried to completion and 2) much reduced need for material with respect to the traditional initial rate method pioneered by Michaelis and Menten. This application of the progress curve method is different in its goal from those used for determining Michaelis-Menten parameters by fitting the integrated Michaelis-Menten equation to a single and complete kinetic run.[5;6] This paper aims at developing methods and the conditions for obtaining individual rate constants, as accurate as possible, that can then be used to calculate Michaelis-Menten parameters, which agree with those determined by other methods.

Our interest in using DYNAFIT has been the elucidation of elementary rate constants in two and three-step consecutive reactions as exemplified by the serine hydrolase-catalyzed hydrolysis of chromogenic substrates, peptides and proteins. An accurate knowledge of the mechanism of an enzyme-catalyzed reaction must be based on competent kinetic information at the level of individual rate constants reporting on specific elementary steps. The slowest elementary step(s) determines the rate of a multi-step process.[7] Once the elementary rate constants are calculated, the isotopic sensitivity of each step can be studied directly. Such direct determination of intrinsic isotope effects is indeed a great gain in enzyme mechanistic studies. Characterization of the transition state(s) of the rate-determining step(s) is at the heart of enzyme mechanistic studies and it is a prerequisite to rational drug design of transition state analog inhibitors.

It is generally expected that the rate-determining step in enzyme-catalyzed ester hydrolysis is deacylation, while that of amide hydrolysis is predominantly acylation.[7;8] The latter premise is often on soft grounds.[8] An elucidation of some of the elementary steps, possibly acylation and deacylation, often requires extensive kinetic studies including trapping of the acyl enzyme or inducing a change in the rate-determining step with changing solvent viscosity,[9–13] salt or temperature.[14;15] The extensive experimental work followed by multidimensional analysis of the results can be abrogated if a reliable computational method can be used for the analysis of a well-designed single determination of the entire reaction progress. However, the uniqueness of calculated rate constants and the robustness and limitations of such a method needs to be proven: Fersht[7] called attention to a caveat that different sets of rate constants may satisfy multi-parameter kinetic equations. The validation of uniqueness of calculated rate constants is thus essential.

Our initial trials using DYNAFIT for fitting a three-step reaction scheme indicated the power of the program and held promise for obtaining reliable individual rate constants of a consecutive reaction scheme, provided a circumspect strategy for analysis is developed. In this paper we report the development of such a strategy. We validated its results against experimentally-determined enzyme kinetic rate constants in the thrombin-catalyzed hydrolysis of H-D-Phe-Pip-Arg-4-nitroanilide.HCl (pNA) (S-2238).[11] The elementary rate constants obtained are in good agreement with the published values. We determined elementary rate constants for three other serine protease-catalyzed reactions that have not before been determined. We probed the robustness of elementary rate constants by varying concentrations of thrombin and activated protein C (APC) and at varying levels of saturation of thrombin and APC, with a chromogenic substrate of medium efficiency for each. The value of the rate constants for the second and third step of the reactions were generally within 3–4-fold. The Michaelis-Menten

constants obtained from a few progress curves reproduced the Literature values reasonably well. We then applied the validated method for exploring full and partial solvent isotope effects on the individual rate constants for the Factor Xa-catalyzed hydrolysis of N- α -Z-D-Arg-Gly-Arg-pNA·2HCl (S-2765). The kinetic rate constants calculated using DYNAFIT are nearly equal for the second and third consecutive steps, corresponding either to the formation and breakdown of the tetrahedral intermediate or to acylation and deacylation, in buffered water. A gradual increase in D₂O content in the buffer resulted in reduced rate constants, mostly for the third consecutive step, moderately for the binding step and only slightly affected the second consecutive step.

Materials and Methods

Materials

Anhydrous dimethyl sulfoxide (DMSO), heavy water with 99.9 % deuterium content and anhydrous methanol, were purchased from Aldrich Chemical Co. All buffer salts were reagent grade and were purchased from either Aldrich, Fisher, or Sigma Chemical Co. H-D-Phe-Pip-Arg-4-nitroanilide.HCl (pNA) (S-2238) 99% (TLC), and Pyro-Glu-L-Pro-L-Arg-pNA.HCl (S-2366) 99% (TLC), were purchased from Diapharma Group Inc.. Human α -thrombin, MM 36,500 d, 3010 NIH u/mg activity in pH 6.5, 0.05 M sodium citrate buffer, 0.2 M NaCl, 0.1% PEG-8000 and Human activated protein C, (APC) MM 56,000 d, 1.19 mg/mL in pH 7.4, 0.02 M Tris-HCl buffer, 0.1 M NaCl M were purchased from Enzyme Research Laboratories. Rabbit thrombomodulin (TM), MM 74,000, 4.7 mg/ml in TBS buffer, 0.05% PDOC, 0.02% NaN₃ was purchased from Haematologic Technologies Inc..

Instruments

Spectroscopic measurements were performed with a Perkin-Elmer Lambda 6 UV-Vis Spectrophotometer connected to a PC. The temperature was monitored using a temperature probe connected to a digital readout device. Either a Neslab RTE-4 or a Lauda 20 circulating water bath was used for temperature control. Positive displacement Gilson and Rainin Microman pipettes with plastic tips were used for the delivery of enzyme solution, substrate solution and organic solvent.

Solutions

Buffers were prepared by weight from Tris-base and Tris-HCl at 0.02 M or 0.005 M Tris, 0.3 M NaCl, 5 mM CaCl₂, 0.1% PEG4000 at pH 8.0 for the experiments with thrombin and APC. The experimental details of the proton inventory studies with of the FXa-catalyzed hydrolysis of N- α -Z-D-Arg-Gly-Arg-pNA·2HCl have been described previously.[16]

Stock solutions for enzymes were prepared by dilution with Tris buffer to 2.90×10^{-7} M thrombin, 7.70×10^{-7} M and 20.8×10^{-7} M APC. Substrate solutions were prepared in DMSO at 2.5×10^{-3} M S-2238 and 0.01 M S-2366.

Enzyme active-site assays

Thrombin concentrations were determined from 20 μ L of a fivefold dilution of the thrombin stock solution, which was added to 960 μ L buffer in a cuvette. The solution was incubated in pH 8.0, 0.02 M Tris buffer, 0.3 M NaCl at 25.0 ± 0.1 °C for 15 minutes. Twenty microliters of 2.5×10^{-3} M S-2238 was added to the solution. Initial rates were measured at 405 nm after quick mixing of the solution. The active-site concentrations were calculated from the velocity measured for a minute and divided by $k_{\text{cat}} = 95 \text{ s}^{-1}$. [17;18] The k_{cat} value was measured under the conditions of these experiments and was based on active-site titration of thrombin with 4-methyl umbelliferyl guanidinobenzoate HCl (MUGB). APC activity was assayed using S-2366

and $k_{\text{cat}} = 160 \text{ s}^{-1}$. [19] There was no background hydrolysis of the substrates in the absence of enzyme and the slopes were proportional to enzyme concentration within 90 % of the value given by the provider.

Kinetic methods

Fifteen microliters of a $1.49 \times 10^{-7} \text{ M}$ thrombin solution was added to 965 μL buffer, pH 8.0, 0.005 M Tris, NaCl 0.2 M, 0.1% PEG4000, and the mixture was incubated for 15 min. Twenty microliters of $2.5 \times 10^{-3} \text{ M}$ S-2238 stock solution in DMSO, to give $5 \times 10^{-5} \text{ M}$ in the cell, was added to initiate the reaction. Completion of a full curve was in 8 minutes. The protocol was nearly identical for the hydrolytic reactions of S-2366 catalyzed by thrombin and APC (Results).

For the proton-inventory study, [16] nine buffers pH 8.4 Tris (and equivalent pL), 0.02 M Tris, 0.3 M NaCl, $5 \times 10^{-3} \text{ M}$ CaCl_2 , 0.1% PEG-4000 were prepared with deuterium oxide composition ranging from atom fraction of D, n, 0 to 99. Ten microliters of $7.23 \times 10^{-7} \text{ M}$ FXa was added to 480 μL of buffer in a cuvette, to give a final concentration of $1.45 \times 10^{-8} \text{ M}$. The cuvette was incubated at $25.0 \pm 0.1^\circ \text{C}$ for 15 min. Ten microliters of 0.05 M S-2765 in DMSO was added to achieve 10^{-3} M in the incubated FXa solution. The measurement was conducted at 445 nm for about 40 min.

Determination of the molar absorptivity of 4-nitroaniline at 405 nm, 445 and 458nm

The molar absorptivity of 4-nitroaniline was determined for the calculation of precise rate constants. The stock solution of 4-nitroaniline was prepared at $9.05 \times 10^{-3} \text{ M}$ in DMSO for the measurement at 445 nm and 458 nm, and at $4.53 \times 10^{-4} \text{ M}$ for 405 nm. pH 8.4 Tris buffer in 460 μL volume was incubated in a cuvette at $25.0 \pm 0.1^\circ \text{C}$ for 15 min. 4-Nitroaniline stock solution was added to the buffer in 10 μL increments of 60 μL in total. The absorbance was recorded after each addition. Calibration curves of absorbance versus concentration were constructed.

Data Analysis

The kinetic data were processed using DYNAFITV, which integrates the requisite simultaneous first-order, nonlinear, ordinary differential equations numerically, using a modification of the multidimensional Newton-Raphson method. [1] Regression analysis in DYNAFIT is performed by Reich's variation of the Levenberg-Marquardt least-squares fitting algorithm. Convergence criteria are multiple in DYNAFIT: The Marquardt parameter for individual parameters should be met first. This was typically met within 100 iterations and 50 subiterations. Standard errors of the individual parameters were computed from the square roots of diagonal elements of the final variance-covariance matrix. The protocol that gave individual rate constants and Michaelis-Menten parameters calculated from them, in agreement with experimentally determined individual rate constants and experimentally determined (or published) Michaelis-Menten parameters, was as follows: Fitting started with a simple two-step consecutive mechanism:

$$\begin{aligned} d[\text{E}]/dt &= -k_1[\text{E}][\text{S}] + k_{-1}[\text{EP}] + k_2[\text{EP}] \\ d[\text{S}]/dt &= -k_1[\text{E}][\text{S}] + k_{-1}[\text{EP}] \\ d[\text{EP}]/dt &= +k_1[\text{E}][\text{S}] - k_{-1}[\text{EP}] - k_2[\text{EP}] \\ d[\text{P}]/dt &= +k_2[\text{EP}] \end{aligned}$$

The initial guesses were based on published values of k_{cat} , which was used for the estimation of k_2 and the concentration of enzymes and substrates were as determined independently. Alternatively, an initial k_{cat} was calculated from the first initial slope of the progress curve (while, $[S] > 4K_m$), V_{max} , and the enzyme concentration, $[E]$, and then used as an estimate of k_2 . The rate constants obtained and offset values, which incur from initial imbalance in absorbance values between reference and sample cells, were then the input for a refined calculation in which the enzyme and substrate concentrations were also calculated. The calculated values of $[E]$ and $[S]$ were within a few percent of the values determined independently in nearly all cases, which is a good indication of program performance when fitting six parameters. In the third round, k_1 , k_{-1} ($K_1 = k_1/k_{-1}$), k_2 and k_3 were calculated for individual runs of 900–3,000 absorption-time data pairs based on a three-step consecutive mechanism:

$$\begin{aligned} d[E]/dt &= -k_1[E][S] + k_{-1}[ES] + k_3[EP] \\ d[S]/dt &= -k_1[E][S] + k_{-1}[ES] \\ d[ES]/dt &= +k_1[E][S] - k_{-1}[EP] - k_2[ES] \\ d[EP]/dt &= +k_2[ES] - k_3[EP] \\ d[P]/dt &= +k_3[EP] \end{aligned}$$

Two, three or four parallel runs were then combined in a "global fit" using individual $[E]$ and $[S]$ values calculated and the values obtained from fits of individual kinetic runs used as initial estimates in the final calculation of the rate constants within the 95% confidence limit. From the three-step reaction scheme, $k_{\text{cat}} = k_2k_3 / (k_2 + k_3)$ and $k_{\text{cat}}/K_m = k_1k_2 / (k_{-1} + k_2)$ were calculated for verification purposes with measured or published values.

Michaelis-Menten parameters were also calculated by fitting the integrated Michaelis-Menten equation:[16]

$$((A - A_0)/\varepsilon k_{\text{cat}}[E]) + K_m/\varepsilon k_{\text{cat}}(\ln(A_{\text{inf}} - A_0)/(A_{\text{inf}} - A)) = t \quad (1)$$

where A , A_0 and A_{inf} are the absorbance values at time t , 0 and infinity, respectively; ε is the molar absorptivity, $[E]$ is the enzyme concentration, and k_{cat} and K_m are the Michaelis-Menten parameters; to the data from the thrombin-catalyzed hydrolysis of S-2238 and the Factor Xa-catalyzed hydrolysis of S-2675 obtained in nine mixtures of water and heavy water buffered at pH 8.2. Good initial estimates of the three parameters are required in the input for the nonlinear fitting here as well. The k_{cat} values agreed within 12 % and the k_{cat}/K_m values agreed within 15 % between the two methods. These and other data reductions using predefined and custom-defined equations were performed using the GraFit 3.0 or GraFit 5.0 software.[20]

Results and Discussion

Molar absorptivities

The molar absorptivities for 4-nitroaniline at pH 8.4, 25.0 ± 0.1 °C and above the 0.998 confidence level are $9,420 \pm 100$, $1,245 \pm 15$ and 346 ± 8 OD $\text{cm}^{-1} \text{M}^{-1}$ at 405, 445 and 458 nm, respectively. A very careful measurement[21] at 405 nm resulted $10,040$ OD $\text{cm}^{-1} \text{M}^{-1}$ in pH 7.8 Tris buffer without PEG4000, which is 6% higher than our value.

Reproducibility of elementary rate constants and Michaelis-Menten parameters calculated from them

As with other nonlinear least squares algorithms, there is a need for good estimates for the parameters to guide the calculation toward convergence at the global minimum, i.e. at correct values. [22;23] This of course is not a serious flaw because one always has access to good estimates as described above and below in this paper. The reproducibility of individual rate constants from individual runs under the same condition, as shown in Tables 1, 2 and 3, is generally very good. The least-squares surface is relatively flat in these calculations, which requires a few repeated restarts of the algorithm after it converged to the Levenberg-Marquardt criteria, in order to bring down the errors. However, the nominal values of the parameters stay stable during these refinements. The calculations obtained from combining three parallel runs provide elementary rate constants with standard deviations below 10% for most cases, and in many instances even below a percent. In Table 1, the standard error for each parameter is reported for each individual run. The benefit of using all data in a global fit is clearly discernable when comparing the standard errors of rate constants obtained from individual runs with the corresponding data from the global fit for any of the elementary rate constants. The same trends are observable for the data presented in Tables 2–5, although the standard errors are not all shown. Typically, the errors in individual rate constants are typically 10–20%, while the global fit gives up to 10% error in the elementary rate constants.

As stated above, experimentally-determined elementary rate constants are not readily available for most complex enzyme-catalyzed reactions. A good agreement between Michaelis-Menten parameters that were calculated from initial rates and those calculated from individual rate constants may be one criterion for the quality of the individual rate constants. The Michaelis-Menten parameters calculated from the value of the optimized elementary rate constants in Table 1, whether with the assumption that k_2 and k_3 are comparable or that $k_2 < k_3$, are reasonably close to the values reported for human thrombin-catalyzed hydrolysis of S-2238. [11;17;18] The parameters for the three other cases also compare well with those reported earlier [16;18;19] and/or the parameters in the Diapharma catalogue measured at a pH near 8.3, at the plateau of the pH profile for these reactions at 37 °C. However, a good agreement between Michaelis-Menten parameters obtained from initial rate measurements and those calculated from progress curves as described here, is not enough guarantee for the accuracy of individual rate constants from which they were calculated, because of the potential distortions due to interdependence of the individual rate constants. The critical question is how well can the concentration of transients be defined in the calculation for the determination of individual rate constants. The greatest uncertainty is in the value of k_1 and k_{-1} , while their ratios are well reproduced. The partitioning of EP to ES versus E + P is the other key element of the calculation.

Validation of elementary rate constants

For this purpose, we chose to study the thrombin-catalyzed hydrolysis of S-2238 at pH 8.0, 0.005 M Tris, 0.2 M NaCl, 0.1% PEG4000 and 25.0 ± 0.1 °C as described in a benchmark study of Wells and Di Cera[11] and at $[S] = 7.5$ K_m. Figure 1 presents the progress curves. Wells and Di Cera[11] used temperature and salt perturbation to change the rate-limiting step and thereby unraveled the individual rate constants for this system. In our progress curve studies, thrombin was highly saturated with S-2238 under the exact same conditions as in the Wells and Di Cera study. As Table 1 illustrates, the value of k_1 , k_{-1} , k_2 and k_3 calculated with DYNAFIT agree within 60% with the values measured by a completely different method. The agreement is even better between the Michaelis-Menten parameters: the best is for k_{cat}/K_m , while both our calculated k_{cat} and K_m values are higher by 60 and 120%, respectively, than the values published by Wells and Di Cera (but in very good agreement with the values listed in the Diapharma catalogue). This deviation is not at all uncommon between two laboratories even when using identical methods. Both experiments predict the second step (k_2) determining

the overall rate of the reaction at 73–76%. We consider this a significant result lending confidence to the individual rate constants which were calculated with DYNAFIT using a circumspect protocol.

Testing the effects of enzyme concentration and levels of enzyme saturation by substrate on the elementary rate constants

We included two enzyme-catalyzed reactions of S-2366, a frequently used substrate of APC and a non-ideal substrate of thrombin, for which individual rate constants have not yet been reported. Enzyme concentrations were varied in some of the repeats, which gave different curves displayed on Figures 2 and 3, but had essentially no effect on the calculated rate constants (Table 2, trials 1 and 2, and Table 3, trials 1–3). These experiments probe then the sensitivity of the calculation to the concentrations of transient intermediates, which vary with the concentration of the enzyme.

Because the K_m values for these reactions are near millimolar, full saturation of the enzymes is not attainable unless co-solvents are used at $> 1\%$. The absorption values at 405 nm, the absorption maximum of pNA, are also prohibitively high at these values of $[S]$. Measurements were carried out at $[S] \sim K_m$ for thrombin and at $[S] \sim 0.125 K_m$ for APC as shown in Figures 2 and 3. A small reduction in the concentration of S-2366 in Table 2, trials 3 and 4, effected the calculated values of all rate constants. The values of k_{cat} and K_m calculated from these individual rate constants are two-fold greater than those reported under very comparable conditions in the Diapharma catalogue, whereas the ratio, k_{cat}/K_m , remain reasonably reproducible. The observation that it is the k_{cat}/K_m ratio rather than individual values of k_{cat} and K_m that is well reproduced at such low concentrations of substrate confirms previous experience in enzyme kinetics. In case of the APC-catalyzed hydrolysis of S-2366 at low substrate concentrations, the Michaelis-Menten parameters are well reproduced.

We studied these two systems in the presence of 10% DMSO, which allowed for raising the concentration of S-2366 to $[S] \sim 6 K_m$ for thrombin and above K_m for APC. The release of pNA was monitored at a shoulder at 445 nm. Figures 4 and 5 show the data obtained and Tables 4 and 5 list the individual rate constants calculated. As might be expected, all rate constants have been affected by the presence of 10% DMSO. In this case, the calculated k_{cat} value for the thrombin-catalyzed reaction agrees well with the one reported earlier, but the K_m value increased, which might have been predicted. The APC-catalyzed hydrolysis of S-2366 in the presence of 10% DMSO again gives both an increased k_{cat} and K_m value, while their ratio agrees with the one measured earlier at 1% DMSO and with the Literature value

Noteworthy is the consistency of the individual rate constants for the thrombin-catalyzed reactions. The bimolecular rate constant, k_1 , is greater for the hydrolysis of S-2238 than for S-2366, which is consistent with the well-documented differences in their affinities for thrombin. Binding is much weaker with S-2366 than with S-2238, which is further supported by the greater rate constants (k_{-1} & k_2) for the decomposition of the Michaelis-Menten complex formed with S-2366. However, both k_2 and k_3 are greater for S-2366 than for S-2238, which is consistent with the relative magnitude in the measured k_{cat} values. Calculated and experimentally measured k_2 and k_3 for S-2238 agree well (Table 1).

Isotopic sensitivity of individual rate constants: intrinsic deuterium isotope effects

One of our major interests is in finding a reliable method for the determination of intrinsic deuterium kinetic isotope effects from proton inventory measurements[16;17;24–33] on the rate-determining step involving proton transfer(s) in hydrolase-catalyzed reactions. In the proton inventory method, kinetic measurements are conducted in mixtures of buffered light and heavy water and the obtained rate constants of such measurements are then plotted against

the atom fraction of deuterium in the solution. A proton inventory study of the 1.45×10^{-8} M FXa-catalyzed hydrolysis of 1×10^{-3} M S-2765 in pH 8.4 and equivalent pL (L = H, D and their mixtures), 0.02 M Tris, 0.3 M NaCl, 5×10^{-3} M CaCl_2 , and 0.1% PEG4000, at 25.0 ± 0.1 °C, resulted in the progress curves in Figure 6 and Table 6 has the calculated rate constants. The initial estimate for k_2 was from k_{cat} , which was calculated from V_{max} given by the slope using the first 10 data points. In the first round of calculations using DYNAFIT, we fitted the two-step model to the data. The calculated rate constants returned from these fits were the estimate for the next round of calculations using the three-step model. The initial estimate of k_3 was varied around the value of k_2 within a range of ten and the final values of all rate constants are in Table 6. The k_{cat} values calculated with the refined values of the elementary rate constants obtained with DYNAFIT are ~20% higher (Table 6) than the initial estimate for all nine cases, which is as expected when enzyme saturation with [S] is extrapolated to infinity. The Michaelis-Menten parameters in aqueous buffer, calculated from the data in Table 6 are: $k_{\text{cat}} = 276 \pm 19 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = (3.9 \pm 0.02) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which compare well with the values obtained from fitting the integrated Michaelis-Menten equation to the time course of two thousand absorption readings: $k_{\text{cat}} = 247.0 \pm 6.2 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = (4.49 \pm 0.32) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. [16] Michaelis-Menten parameters were also calculated from the elementary rate constants for the other eight data sets obtained in varying isotopic buffer mixtures and are compared in Table 7 with the corresponding values calculated with the integrated Michaelis-Menten equation. The agreement is good, as it is good with $k_{\text{cat}} = 240 \text{ s}^{-1}$, $K_m = 2.6 \times 10^{-4}$ M, and $k_{\text{cat}}/K_m = 9.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in pH 8.3, 0.05 M Tris, 0.13 M NaCl and 0.5% BSA at 37 °C measured in the Diapharma lab.

Gross and Butler described the equation relating rates or rate constants to deuterium content in solution as follows:[24;27;28;34;35]

$$V_n = V_o \prod_i^{\text{TS}} (1 - n + n\phi_i^{\text{T}}) / \prod_j^{\text{RS}} (1 - n + n\phi_j^{\text{R}}) \quad (2)$$

where V_n and V_o are velocity (or rate constant) in a binary solvent and in water, respectively, n = atom fraction of deuterium, RS = reactant state, TS = transition state, ϕ^{R} = RS fractionation factor and ϕ^{T} = TS fractionation factor. The fractionation factors are obtained from inverted equilibrium isotope effects, K_D/K_H , for exchange between a bulk water site and a particular structural site of the RS or the TS. Justifiable simplifications of this equation involve the assumption of a unit fractionation factor of RSs for catalytic residues with NH and OH functional groups and the assumption that one or two active-site units contribute in most hydrolytic enzymes.

Figure 7 shows the proton inventory curves: The equations of the lines for fitting the data are modifications,[16;17;33] of the Gross-Butler equation:[24;27;28;34;35] $k_n/k_H = (1 - n + n/(2.0))$ for K_1 , $k_n/k_H = (1 - n + n/(1.4))$ for k_2 , and $k_n/k_H = (1 - n + n/(3.1)^2)$ for k_3 , respectively.

Rate-determining step

Hedstrom[8] warns against the fallacy in the generally held belief that acylation is slower for amide hydrolysis than deacylation. She arraigns a number of cases where this expectation fails. Many other hydrolase-catalyzed reactions are known for which the rate is determined partly by acylation and partly by deacylation.[36] Although the true identity of k_2 and k_3 has not been revealed in these studies, they were assigned to acylation and deacylation in the thrombin-catalyzed reactions of S-2238[11] and in reactions of other substrates when studied with the perturbation techniques cited in the Introduction.[10;12–15] By analogy, we may assign the k_2 and k_3 values for the thrombin-catalyzed hydrolysis of S-2238 also to acylation and deacylation, respectively. We then extend this choice to the other cases too: k_2 and k_3 measure

acylation and deacylation, respectively. The DYNAFIT-calculated values of k_2 and k_3 are never distinctly different, in fact, they are nearly equal for the reactions of S-2366 and for the last case, the FXa-catalyzed hydrolysis of S-2675. As discussed above, this is often the case experimentally.

Of great importance is that the value of k_2 is insensitive to the content of D, while k_3 decreases dramatically as the D content is increased in the buffer system, everything else being the same. The third step, quite likely the hydrolysis of the acyl enzyme, becomes increasingly rate determining with increasing D content in the medium. The proton inventory curve (Figure 7) is deeply concave and is most consistent with two protonic bridges participating in deacylation with fractionation factors 0.32 ± 0.02 each and corresponding to an intrinsic isotope effect of 3.1 ± 0.2 . The protonic bridges are most likely to be between the catalytic Ser and His and between His and Asp at the active site.[17;33] The proton inventory for the binding step and acylation step are nearly linear within experimental errors and the fractionation factors associated with them are smaller in value and less certain in origin.

This is a very significant result, because the method allows for the determination of the intrinsic solvent isotope effects or partial solvent isotope effects of individual steps of a complex reaction. An access to intrinsic deuterium isotope effects in the rate-determining step of enzyme-catalyzed acyl transfer reactions will be a major advance in the interpretation of the role of proton transfer in these mechanisms.

In conclusion, the use of DYNAFIT holds the premise for obtaining individual rate constants from a relatively small number of, albeit meticulously precise, progress curve measurements for irreversible one substrate reactions without substrate and product inhibition. Even poor substrates that may not be used in concentrations that saturate the active sites give reasonable results.

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Figure 1.

Three progress curves for 1.49×10^{-9} M human thrombin-catalyzed hydrolysis of 4×10^{-5} M S-2388 at pH 8.0, 0.005 M Tris, 0.2 M NaCl, 0.1% PEG4000 and 25.0 ± 0.1 °C, monitored at 405 nm. Symbols indicate selected calculated points.

Figure 2.

Progress curves of human thrombin-catalyzed (IIa) hydrolysis of S-2366 (S) under non-ideal conditions ($[S] \sim K_m$) in the presence of thrombomodulin in pH 8.0, 0.02 M Tris, 0.3 M NaCl, 0.1% PG4000 and 25.0 ± 0.1 °C: 1) $[IIa] = 11.6 \times 10^{-9}$ M, $[S] = 2.00 \times 10^{-4}$ M ; 2) $[IIa] = 3.9 \times 10^{-9}$ M, $[S] = 2.00 \times 10^{-4}$ M; 3) and 4) $[IIa] = 3.9 \times 10^{-9}$ M, $[S] = 1.32 \times 10^{-4}$ M, monitored at 405 nm. The calculated and measured data points are indistinguishable on this scale.

Figure 3. APC-catalyzed hydrolysis of 1.00×10^{-4} M S-2366 ($[S] = 0.125 K_m$) in pH 8.0, 0.02 M Tris, 0.3 M NaCl, and 0.1% PEG4000, at 25.0 ± 0.1 °C, monitored at 405 nm: [APC] 1) 1.54×10^{-8} M, 2) 3.08×10^{-8} M and 3) 4.62×10^{-8} M.

Figure 4.

Three progress curves for the 7.9×10^{-9} M thrombin-catalyzed hydrolysis of S-2366, at $[S] = 6 K_m$, in pH 8.0, 0.02 M Tris, 0.3 M NaCl, 0.1% PG4000, 25.0 ± 0.1 °C, but in the presence of 10% DMSO, and monitored at 445 nm. Symbols indicate selected calculated points.

Figure 5.

The 4.15×10^{-8} M APC-catalyzed hydrolysis of S-2366 at $[S] = 1.2 K_m$, in pH 8.0, 0.02 M Tris, 0.3 M NaCl, 0.1% PG4000, 25.0 ± 0.1 °C but in the presence of 10% DMSO, and monitored at 445 nm. Symbols indicate calculated points.

Figure 6.

Progress curves for the 1.45×10^{-8} M FXa-catalyzed hydrolysis of 1×10^{-3} M S-2765 in pH 8.4 and equivalent pL (L = H, D and their mixtures), 0.02 M Tris buffer, 0.3 M NaCl, 5×10^{-3} M CaCl_2 , 0.1% PEG4000, at 25.0 ± 0.1 °C and monitored at 445 nm. All runs were followed to completion (not all shown on the figure).

Figure 7.

Proton Inventory for $K_1 = k_1/k_{-1}$, triangles, k_2 closed circles, and k_3 open circles. The equations of the lines for fitting the data were modifications,[17;31] of the Gross-Butler equation;[22; 25;26;32;33] $k_n/k_H = (1-n + n/(2.03))$; $k_n/k_H = (1-n + n/(1.43))$; and $k_n/k_H = (1-n + n/(3.11)^2)$, respectively.

Table 1

Rate constants calculated with the two- and three-step model for individual and combined runs for the 1.49×10^{-9} human thrombin-catalyzed hydrolysis of 4×10^{-5} MS-2238 at pH 8.0, 0.005 M Tris buffer, 0.2 M NaCl, 0.1% PEG4000 and 25.0 ± 0.1 °C

Parameter	Individual trials, three steps			Global fit 1+2+3		Ref.[11]	Diapharma ^a
	1	2	3	Two steps	Three steps		
$k_1, \mu\text{M}^{-1}\text{s}^{-1}$	66.0 ± 8.7	67.3 ± 4.0	64.4 ± 7.9	62.0 ± 5.0	64.2 ± 1.8	103 ± 28	
k_{-1}, s^{-1}	448 ± 73	423 ± 77	417 ± 81	249 ± 33	420 ± 26	280 ± 29	
k_2, s^{-1}	187 ± 14	183 ± 24	183 ± 17	150.00 ± 0.02	185 ± 9	101 ± 10	
k_3, s^{-1}	400 ± 65	409 ± 120	414 ± 90	-	404 ± 43	315 ± 63	
$k_{\text{cat}}, \text{s}^{-1}$	127 ± 23	126 ± 40	127 ± 30	150.00 ± 0.02	127.0 ± 16.0	77 ± 3	$180, 95^b$
$K_m, \mu\text{M}$	6.5 ± 1.9	6.2 ± 2.4	6.4 ± 2.1	6.43 ± 0.84	6.47 ± 1.0	2.8 ± 0.3	7.0
$k_{\text{off}}/K_m, \text{M}^{-1}\text{s}^{-1}$	19.4 ± 4.1	20.0 ± 4.4	19.7 ± 4.5	23.3 ± 3.1	19.6 ± 1.6	27.5	26.7

^a pH 8.3, 0.05 M Tris, and 0.13 M NaCl at 37 °C.

^b Conditions as above [17;18]

Rate constants calculated from individual and combined runs for the human thrombin-catalyzed S-2366 hydrolysis (see Figure 2) in the presence of thrombomodulin in pH 8.0, 0.02 M Tris buffer, 0.3 M NaCl, 0.1% PG4000 and 25.0 ± 0.1 °C

Parameter	Individual trials, three steps			Global fit 1+2+3			DiaPharma ^a
	1	2	3	4 [*]	Two steps	Three steps	
k ₁ , μM ⁻¹ s ⁻¹	8.01	7.55	8.13	8.17	8.33 ± 0.44	7.92 ± 0.6	-
k ₋₁ , s ⁻¹	6970	7340	6670	6630	3010 ± 160	6910 ± 680	-
k ₂ , s ⁻¹	2070	1950	2110	2140	800 ± 43	2043 ± 130	-
k ₃ , s ⁻¹	1760	1130	1410	4760	-	1077 ± 92	-
k _{cat} , s ⁻¹	952	715	846	1477	800 ± 43	705 ± 83	330
K _m , μM	519	451	433	740	457 ± 31	390 ± 67	150
k _{cat} /K _m , μM ⁻¹ s ⁻¹	1.84	1.58	1.95	2.00	1.76 ± 0.15	1.81 ± 0.23	2.2

* Outlier

^a pH 8.3, 0.05 M Tris, 0.13 M NaCl at 37 °C.

Rate constants calculated from individual runs and combined data for the human APC-catalyzed hydrolysis of S-2366, in pH 8.0, 0.02 M Tris buffer, 0.3 M NaCl, and 0.1% PEG4000, at 25.0 ± 0.1 °C (Figure 3)

Table 3

Parameter	Individual trials, three steps			Global fit 1+2+3		Ref [19] *
	1	2	3	Two steps	Three steps	
10 ⁸ [APC], M	1.54	3.08	4.62			
k ₁ , μM ⁻¹ s ⁻¹	3.61	3.50	3.58	4.17 ± 0.30	3.54 ± 0.20	-
k ₋₁ , s ⁻¹	8560	8650	8540	2850 ± 230	8580 ± 390	-
k ₂ , s ⁻¹	593	586	594	159 ± 13	590 ± 33	-
k ₃ , s ⁻¹	170	297	227	-	229 ± 11	-
k _{cat} , s ⁻¹	132	197	164	159 ± 13	165 ± 14	160
K _m , μM	571	883	708	723 ± 76	723 ± 90	800
k _{cat} /K _m , μM ⁻¹ s ⁻¹	0.231	0.223	0.232	0.220 ± 0.029	0.228 ± 0.021	0.2

* pH 8.0, 0.05 M Tris buffer, 0.13 NaCl and 0.01 M CaCl₂ at 25.0 °C.

Table 4

Rate constants calculated from individual and combined data for the 7.90×10^{-9} M human thrombin-catalyzed hydrolysis of S-2366 at $[S] = 6 K_m$, as described in Table 2 but in the presence of 10% DMSO

Parameter	Individual trials, three steps			Global fit Two step		Global fit Three step	
	1	2	3				
$k_1, \mu M^{-1} s^{-1}$	1.56	1.65	1.73	2.42 ± 0.09	1.87 ± 0.1		
k_{-1}, s^{-1}	2680	2660	2530	1775 ± 58	2294 ± 160		
k_2, s^{-1}	868	883	892	372 ± 17	717 ± 56		
k_3, s^{-1}	719	677	543	-	877 ± 78		
k_{cat}, s^{-1}	393	383	338	372 ± 17	395 ± 52		
$K_m, \mu M$	1028	933	749	887 ± 40	886 ± 153		
$k_{cat}/K_m, \mu M^{-1} s^{-1}$	0.382	0.411	0.450	0.420 ± 0.027	0.445 ± 0.05		

Calculated rate constants from individual and combined runs for human APC-catalyzed hydrolysis of S-2366 at 1.2 K_m as indicated in Table 3, but in the presence of 10% DMSO

Table 5

Parameter	Individual trials, three steps			Global fit Two step	Global fit Three step
	1	2	3		
$k_1, \mu\text{M}^{-1}\text{s}^{-1}$	3.20	3.19	3.19	2.65 ± 0.05	3.20 ± 0.04
k_{-1}, s^{-1}	9440	9450	9480	4200 ± 92	9488 ± 420
k_2, s^{-1}	560	555	554	290 ± 3	556 ± 22
k_3, s^{-1}	914	606	584	-	651 ± 29
$k_{\text{cat}}, \text{s}^{-1}$	347	290	284	290 ± 3	300 ± 20
$K_m, \mu\text{M}$	1940	1640	1620	1690 ± 49	169 ± 151
$k_{\text{cat}}/K_m, \text{M}^{-1}\text{s}^{-1}$	0.179	0.177	0.176	0.171 ± 0.005	0.177 ± 0.01

Table 6

Rate constants calculated using DYNAFIT for proton inventory study of the 1.45×10^{-8} M human FXa-catalyzed hydrolysis of S-2765 = 1×10^{-3} M in pH 8.4 and equivalent pL (L = H, D and their mixtures), 0.02 M Tris buffer, 0.3 M NaCl, 5×10^{-3} M CaCl_2 , and 0.1% PEG4000, at 25.0 ± 0.1 °C

n	$10^6 k_1, \text{M}^{-1}\text{s}^{-1}$	k_{-1}, s^{-1}	k_2, s^{-1}	k_3, s^{-1}
0.00	4.74 ± 0.07	5750 ± 250	521 ± 21	586 ± 27
0.12	4.75 ± 0.16	5789 ± 340	514 ± 40	474 ± 33
0.24	3.98 ± 0.20	6066 ± 470	456 ± 31	364 ± 20
0.37	4.00 ± 0.10	6040 ± 170	457 ± 22	296 ± 9
0.49	4.88 ± 0.13	7619 ± 67	518 ± 15	220 ± 3
0.61	4.24 ± 0.16	6738 ± 420	471 ± 44	175 ± 6
0.73	3.57 ± 0.09	7042 ± 150	387 ± 7	162 ± 1
0.86	3.49 ± 0.07	7603 ± 130	378 ± 9	116 ± 1
0.98	3.29 ± 0.21	7846 ± 110	363 ± 26	94 ± 2

Table 7

A comparison of Michaelis-Menten parameters calculated from the individual rate constants obtained with DYNAFIT in Table 6 with those calculated using eq 1 for the integrated Michaelis-Menten equation. The nine sets of 2000 data pairs were obtained for proton inventory study[16] of the 1.45×10^{-8} M human FXa-catalyzed hydrolysis of S-2765 = 1×10^{-3} M in pH 8.4 and equivalent pL (L = H, D and their mixtures), 0.02 M Tris buffer, 0.3 M NaCl, 5×10^{-3} M CaCl_2 , and 0.1% PEG4000, at 25.0 ± 0.1 °C

n	$k_{\text{cat}}, \text{s}^{-1}$		$10^{-5} k_{\text{cat}}/K_m, \text{M}^{-1}\text{s}^{-1}$	
	Dynafit	Equation 1	Dynafit	Equation 1
0	276 ± 19	247.0 ± 6.2	3.9 ± 0.2	4.49 ± 0.32
0.12	247 ± 29	212 ± 13	3.9 ± 0.4	3.79 ± 0.33
0.24	202 ± 20	181.3 ± 5.5	2.8 ± 0.3	2.81 ± 0.15
0.37	179 ± 12	165.9 ± 1.8	2.8 ± 0.2	2.71 ± 0.18
0.49	154 ± 6	133.2 ± 5.1	3.1 ± 0.1	3.73 ± 0.39
0.61	128 ± 16	115.8 ± 5.1	2.8 ± 0.3	2.85 ± 0.16
0.73	114 ± 3	93.6 ± 1.5	1.9 ± 0.1	2.21 ± 0.13
0.86	88 ± 3	75.9 ± 0.6	1.7 ± 0.1	1.60 ± 0.21
0.98	74 ± 7	66.1 ± 0.1	1.5 ± 0.1	1.52 ± 0.21

Note: Maximal velocities were calculated from the slope given by the first ten data points in each set and an initial value of k_{cat} was calculated from these and [E]. It was assumed that $k_{\text{cat}} = k_2$ for the first round of DYNAFIT calculations. The k_2 value returned from the fit was then used as initial estimate for the next round of fit with the three step model. The estimate for k_3 was varied within ten fold around the value of k_2 . The k_{cat} values calculated from the individual rate constants in the first column are ~ 20% higher than the initial estimates, which is as expected when enzyme saturation with [S] is extrapolated to infinity.