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Isothermal titration calorimetry determination of individual rate constants of trypsin catalytic activity

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ABSTRACT

Determination of individual rate constants for enzyme-catalyzed reactions is central to the understanding of their mechanism of action and is commonly obtained by stopped-flow kinetic experiments. However, most natural substrates either do not fluoresce/absorb or lack a significant change in their spectra while reacting and, therefore, are frequently chemically modified to render adequate molecules for their spectroscopic detection. Here, isothermal titration calorimetry (ITC) was used to obtain Michaelis–Menten plots for the trypsin-catalyzed hydrolysis of several substrates at different temperatures (278–318 K): four spectrophotometrically blind lysine and arginine N-free esters, one N-substituted arginine ester, and one amide. A global fitting of these data provided the individual rate constants and activation energies for the acylation and deacylation reactions, and the ratio of the formation and dissociation rates of the enzyme–substrate complex, leading also to the corresponding free energies and enthalpies of activation. The results indicate that for lysine and arginine N-free ester deacylation is the rate-limiting step, but for the N-substituted ester and the amide acylation is the slowest step. It is shown that ITC is able to produce quality kinetic data and is particularly well suited for those enzymatic reactions that cannot be measured by absorption or fluorescent spectroscopy.

Trypsin is a digestive enzyme that belongs to the PA(S) clan and S1 family of serine proteases in the MEROPS database [1]. Its function is to hydrolyze the amide bond of proteins and peptide substrates preferentially at the C terminus of lysine and arginine residues [2]. However, it can hydrolyze other acyl compounds as amides [3,4], anilides [5,6], esters [3,4,7,8], thioesters [9,10], and phosphonates [11]. Its active site possesses the catalytic triad residues Ser195, His57, and Asp102 that are specific of this class of serine proteases. The simplest accepted mechanism of serine protease amide- or ester-catalyzed hydrolysis is depicted in Fig. 1. The first step involves binding of the substrate [S] and enzyme [E] to produce the enzyme–substrate complex [E–S] and is characterized by the kinetic constants k_1 and k_{-1}. It follows the acylation step in which the Ser195 residue attacks the substrate's carbonyl group, forming a covalently bound intermediate [E–Ac] concomitant with the departure of the amine or alcohol group [P1] from the active site. Finally, in the deacylation step, His57 activates a water molecule that attacks the acylenzyme, regenerating the free enzyme and releasing the carboxylic part of the original substrate [P2]. Acylation and deacylation proceed through the formation of short-life tetrahedral intermediates stabilized by the pocket formed by the backbone NHs of residues Gly193 and Ser195, known as the oxyanion hole [12,13]. Although acylation and deacylation are composed of several discrete steps, they are included in single kinetic constants, namely k_2 and k_3 (Fig. 1).

Although trypsin kinetics has been studied extensively [13–15], several questions have not been addressed. One question of particular interest is whether natural peptides or spectroscopically blind substrates (which normally contain poor leaving groups) have the same rate-limiting step as the chemically modified substrates with spectroscopically active leaving groups. The answer to this question can be reached through the evaluation of the individual rate constants (sometimes referred to as microscopic rate constants) depicted in Fig. 1. For the spectroscopically active compounds, measuring the absorption or fluorescence emission of substrates or products in a stopped-flow instrument is probably the most direct way to obtain these rates. However, the majority of commercial peptides or esters for serine proteases with spectroscopically active leaving groups (*p*-nitroaniline, 4-methyl-7-aminocoumarin, *p*-nitrophenol, and 4-methyl-7-hydroxycoumarin [5,16,17]) might be more reactive than the natural substrates, in some cases giving inaccurate information about the limiting steps

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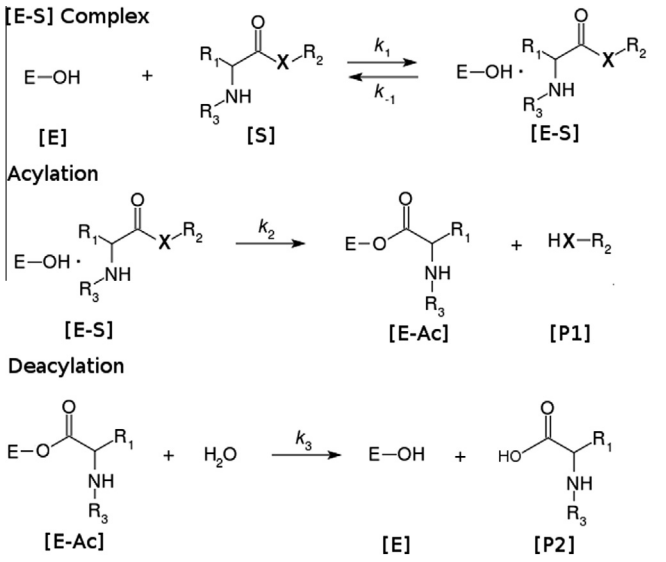

Fig.1. Mechanism of amide/ester hydrolysis catalyzed by serine proteases (X = NH or O).

of the reaction pathway or overestimation of the individual catalytic rate constants with respect to the cognate substrates [18–20]. For the spectroscopically blind substrates, it is possible to determine the individual rate constants by the rapid quench–flow technique [21]. However, the reaction intermediate concentrations need to be determined by another method (e.g., mass spectroscopy [22], liquid scintillation spectrometry [23], pH-stat titration [7,24], liquid chromatography [25]), and because these procedures are discontinuous, many time-consuming experiments would need to be performed to obtain a single time course [21].

Steady-state kinetics data obtained at different temperatures can also be used to determine individual rate constants [26–31]. This method consists of fitting the enzymatic parameters k_{cat} and k_{cat}/K_M acquired at different temperatures to equations where these kinetic constants are expressed in terms of their individual rate constants, whose temperature dependence is assumed to follow the Arrhenius equation. For the case of some serine proteases, this approach has been successfully applied using spectrophotometric assays [27–29]. However, when dealing with spectrophotometrically blind substrates, the isothermal titration calorimetry (ITC)[1] technique is particularly well suited to determine the steady-state kinetic constants [32–34]. ITC can measure the heat released or absorbed during a chemical reaction, a quantity that is directly correlated with the reaction rate, and presents the important advantage that the natural substrates of an enzyme can be used directly without the need for chemical modifications, avoiding the drawbacks mentioned above for modified substrates. This technique also permits a high degree of control of the temperature reaction, and in a single experiment a time course can be obtained and transformed to the usual rate versus substrate concentration Michaelis–Menten plots [32–34]. Nevertheless, the heat transfer in the microcalorimeter is not a fast process and requires a few seconds for the instrument to react to the amount of heat that is being released or absorbed in the reaction cell, preventing ITC from being used in pre-steady-state kinetic assays.

In this report, we used ITC to measure the steady-state kinetics at different temperatures of the promiscuous enzymatic hydrolysis by bovine trypsin of five esters and one amide. We obtained reaction rates (v) versus substrate concentration ([S]) plots for the enzymatic hydrolysis at many different temperatures and then performed a global simultaneous fit of the whole set of data at all temperatures for each substrate. For this fitting, k_{cat} and K_M in the Michaelis–Menten equation were written in terms of the individual rate constants, assuming for them an Arrhenius-type temperature dependency. This allowed the evaluation of the individual rate constants with their respective activation energies. Finally, we determined the free energies of activation ($\Delta G^{\ddagger}$) for each step in the mechanism.

Materials and methods

Chemicals

All substrates (Fig. 2) and bovine pancreatic β-trypsin type III (23.3 kDa) were obtained from Sigma–Aldrich. All other chemicals were reagent grade. Trypsin solution concentrations were determined with a spectrophotometric assay according to established procedures [35,36].

Enzyme kinetic experiments using ITC

Enzyme kinetic experiments were performed on a VP-ITC microcalorimeter (MicroCal) over the temperature range 278.15 to 318.15 K at pH 8.0 in Activity Buffer (200 mM Tris–HCl, 50 mM $CaCl_2$, and polyethylene glycol [PEG] 5000 [0.2%, w/v]). Because the pK_a of Tris changes substantially with temperature, the solutions were heated or cooled to the desired temperature in a water bath and the pH was carefully adjusted to 8.0 using a calibrated pH meter (Orion Star A211, Thermo Scientific). In a typical experiment, trypsin (50 nM) was placed into the calorimetric cell (which has 1.4196 ml of effective reaction volume, measured and reported by the manufacturer), and the syringe (300 µl) was filled with the desired substrate (70 mM). A single injection of 40 µl was made to get a final substrate concentration in the cell of 2.0 mM, and the thermal power signal (µJ/s) was recorded until it returned to the original baseline (reaction completion). The hydrolysis of Lys-pNA was determined by placing the substrate in the calorimetric cell (6.0 mM) and injecting 200 µl of the trypsin solution (0.4 mM). For some systems, triplicate or duplicate experiments were performed. Given the excellent reproducibility, for another group of systems no repetition was carried out. Electrical calibrations are periodically performed in the VP-ITC unit.

Enzyme kinetic experiments with ultraviolet–visible spectroscopy

Initial rates for the trypsin-catalyzed hydrolysis of Bz-Arg-Et and Lys-pNA were measured in Activity Buffer using a CARY-50 spectrophotometer at 253 nm for the first substrate and 410 or 445 nm for the second substrate. Activity Buffer was carefully adjusted at pH 8.0 for each experimental temperature (278.15–318.15 K) as described above. Trypsin final assay concentrations were 10 nM for Bz-Arg-Et and 50 µM for Lys-pNA.

Results

Steady-state kinetic parameters from individual fittings

The measurements of the trypsin-catalyzed hydrolysis of the substrates shown in Fig. 2 generate thermal power versus time graphs such as that shown in Fig. 3A. Using Origin 7.0 software provided by MicroCal, these raw data were corrected by the instrument time response constant (18.47 s) and then converted to

[1] *Abbreviations used:* ITC, isothermal titration calorimetry; PEG, polyethylene glycol; UV–Vis, ultraviolet–visible.

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