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Kinetics of trypsin-catalyzed hydrolysis determined by isothermal titration calorimetry

Ksenia Maximova *, Joanna Trylska *

Centre of New Technologies, University of Warsaw, 02-097 Warsaw, Poland

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ABSTRACT

Isothermal titration calorimetry (ITC) was applied to determine enzymatic activity and inhibition. We measured the Michaelis–Menten kinetics for trypsin-catalyzed hydrolysis of two substrates, casein (an insoluble macromolecule substrate) and Nα-benzoyl-DL-arginine β-naphthylamide (a small substrate), and estimated the thermodynamic parameters in the temperature range from 20 to 37 °C. The inhibitory activities of reversible (small molecule benzamidine) and irreversible (small molecule phenylmethanesulfonyl fluoride and macromolecule α1-antitrypsin) inhibitors of trypsin were also determined. We showed the usefulness of ITC for fast and direct measurement of inhibition constants and half-maximal inhibitory concentrations and for predictions of the mechanism of inhibition. ITC kinetic assays could be an easy and straightforward way to estimate Michaelis–Menten constants and the effectiveness of inhibitors as well as to predict the inhibition mechanism. ITC efficiency was found to be similar to that of classical spectrophotometric enzymatic assays.

Enzyme kinetics is an informative study of enzyme-catalyzed reaction rates. Such studies enable predicting the pathway of the catalyzed reaction and developing potential inhibitors of enzyme activity. The most common laboratory methods to study enzyme kinetics and inhibition are spectrophotometric and fluorometric assays. Although widely used, these techniques are frequently too costly or time-consuming because they require labeling of substrates with the chromophore or fluorophore groups. Moreover, opaque or turbid solutions are inappropriate for spectrophotometric detection, which prevents direct studies of insoluble proteins and presupposes multistep procedures. Isothermal titration calorimetry (ITC)[1] could be used as an alternative technique in such troublesome cases because all reactions proceed with the absorption or release of heat, which ITC is able to detect with straightforward and fast measurements [1–5].

ITC is a physical technique for the estimation of the thermodynamic parameters of binding of small molecules to larger macromolecules [6]. Typically, the ligand is titrated into the cell with a receptor and the thermal power is monitored at each step. Thermal power data can be further analyzed to determine thermodynamic parameters such as enthalpy changes and equilibrium association constants. This technique is widely used to check the binding of inhibitors to their targets [6]. However, the drawback of such binding titration ITC experiments is that they require sufficient amounts of components (in the range of μM to mM), which can be either costly or difficult to obtain. This technique has not been appreciated for the kinetic studies where the amounts are comparable to those required for classical spectrophotometric enzymatic assays (in the range of pM to nM).

There are few studies that present the use of ITC for kinetic measurements, and they show good correlation between calorimetric and spectrophotometric data for reaction kinetics [1,3,7,8]. The ITC approach was applied to determine kinetic parameters for opaque solutions [9], for complex, multi-substrate, and multi-protein systems [10], for reactions with insoluble substrates [11], and to detect reaction inhibition by products and determine the thermodynamic activation parameters [12,13]. However, applications of ITC to enzyme kinetics are still rare, probably because of scarce literature data on this particular application and optimization of the conditions.

We applied the ITC technique to determine the kinetics of trypsin (24 kDa)-catalyzed hydrolysis of casein (24 kDa), which is an example of insoluble macromolecule substrate, and Nα-benzoyl-DL-arginine β-naphthylamide (BANA; 440 Da), which is an example of a small molecule substrate (Fig. 1). We also verified reaction inhibition by products and determined the

* Fax: +48 (22) 5540 801.
E-mail addresses: k.maximova@cent.uw.edu.pl (K. Maximova), joanna@cent.uw.edu.pl (J. Trylska).

[1] *Abbreviations used:* ITC, isothermal titration calorimetry; BANA, Nα-benzoyl-DL-arginine β-naphthylamide; BA, benzamidine; PMSF, phenylmethanesulfonyl fluoride; AT, α1-antitrypsin; DMSO, dimethyl sulfoxide.

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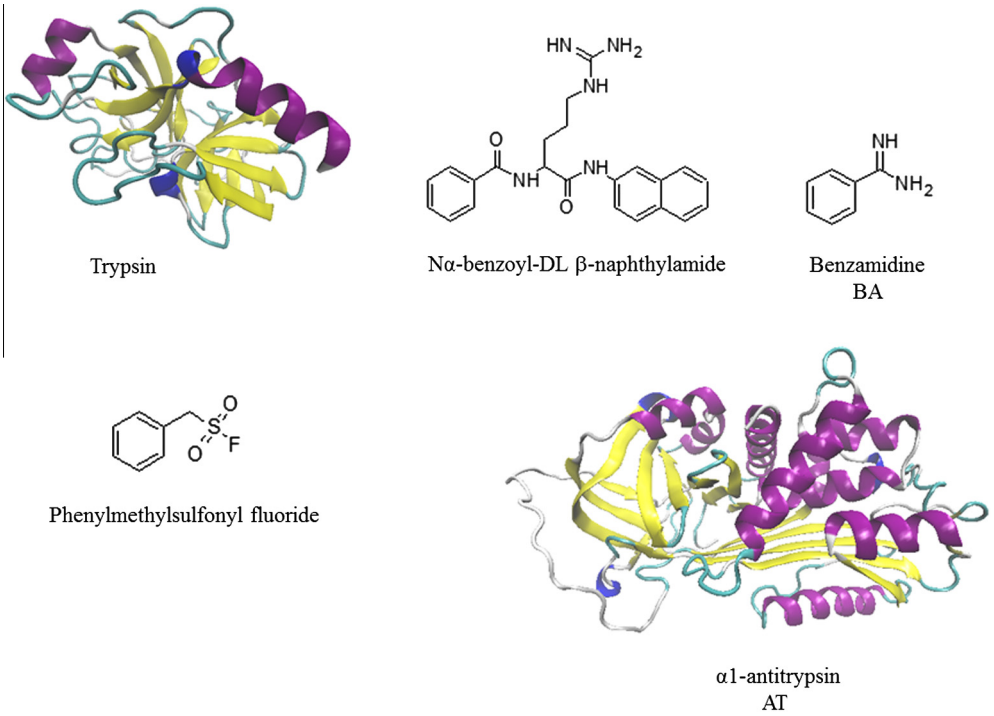

Fig.1. Backbone representation of a bovine pancreatic trypsin (223 amino acids, PDB code: 1S0Q), *N*α-benzoyl-DL-arginine β-naphthylamide (BANA), benzamidine (BA), phenylmethanesulfonyl fluoride (PMSF), and human α1-antitrypsin (424 amino acids, PDB code: 3NE4).

thermodynamic activation parameters. The ITC-based method was also explored to investigate enzyme inhibition by benzamidine (BA; 120 Da), selected as an example of a small molecule reversible competitive inhibitor, phenylmethanesulfonyl fluoride (PMSF; 174 Da), selected as an example of a small molecule irreversible inhibitor, and α1-antitrypsin (AT; 52 kDa), selected as an example of a macromolecule irreversible inhibitor (Fig. 1).

Materials and methods

All chemicals were purchased from Sigma–Aldrich and used without further purification.

Enzyme

The stock solution (1 mM) of trypsin from bovine pancreas (⩾10,000 BAEE units/mg protein) was prepared in 1 mM HCl and stored at −20 °C.

Substrates

The stock solution (70 mM) of BANA was prepared in dimethyl sulfoxide (DMSO). The casein solution of 45.7 mg/ml (1.905 mM) was prepared in 50 mM potassium phosphate buffer at pH 7.5 by gentle heating with stirring to 80–85 °C for approximately 10 min until a homogeneous dispersion was achieved.

Inhibitors

The stock solution (64 mM) of BA was freshly prepared in 50 mM potassium phosphate buffer at pH 7.5. The stock solution (100 mM) of PMSF was freshly prepared in ethanol. The stock solution (0.4 mM) of AT from human plasma was prepared in 1% sodium azide and stored at −80 °C.

The composition of the buffer solution for the substrates and the enzyme, with or without inhibitors, for the assay experiments was identical. The concentration of DMSO in assays with BANA was kept at 10%, and the concentration of EtOH in assays with PMSF was kept at 1%.

All experiments were performed in triplicates in 50 mM potassium phosphate buffer at pH 7.5, and in tables the determined averages are shown. In all cases, the mean percentage deviation from the average was within 15%.

Equipment

The ITC assays were carried out on the power-compensated instruments MicroCal iTC200 (MicroCal, Northampton, MA, USA) and Nano ITC (TA Instruments, New Castle, DE, USA). The MicroCal iTC200 has a Hastelloy cell of 200 µl volume and a syringe of 40 µl volume, and the Nano ITC has a gold cell of 190 µl volume and a syringe of 50 µl volume. The stirring speed in the calorimeter cells was 350 rpm, and thermal power was recorded every 2 s. In each experiment, the control injections of the substrate to the buffer and of the buffer to the enzyme were carried out, and the heat of the dilution was corrected. The MicroCal iTC200 and Nano ITC were used with the control software provided by the manufacturers, MicroCal Origin 7 and NanoAnalyze, respectively.

Enzyme assays for the determination of apparent molar enthalpy ΔH_{app} (Nano ITC and MicroCal iTC200) and kinetic constants (MicroCal iTC200)

Trypsin-catalyzed hydrolysis of casein: single injection assay

The trypsin solution (1.95 µM) in 50 mM potassium phosphate buffer (pH 7.5) was equilibrated at 37 °C for 200 s. Then 1 aliquot of 10 µl of the casein solution (1.9 mM) was injected. The change in the instrumental thermal power was monitored until the substrate hydrolysis was complete, that is, the signal returned to the original baseline (3000 s). Data analysis gave ΔH_{app} = 108 kJ/mol (Nano ITC) and 103.8 kJ/mol (MicroCal iTC200).