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A quenched-flow system for measuring heterogeneous enzyme kinetics with sub-second time resolution

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ABSTRACT

Even though many enzyme processes occur at the interface of an insoluble substrate, these reactions are generally much less studied than homogenous enzyme reactions in the aqueous bulk. Interfacial (or heterogeneous) enzyme reactions involve several reaction steps, and the established experimental approach to elucidate multi-step reactions is transient (or pre steady-state) kinetics. A key requirement for pre steady-state measurements is good time resolution, and while this has been amply achieved in different commercial instruments, they are generally not applicable to precipitating suspensions of insoluble substrate. Perhaps for this reason, transient kinetics has rarely been reported for heterogeneous enzyme reactions. Here, we describe a quenched-flow system using peristaltic pumps and stirred substrate suspensions with a dead time below 100 ms. The general performance was verified by alkali catalyzed hydrolysis of 2,4-dinitrophenyl acetate (DNPA), and the applicability to heterogeneous reactions was documented by two cellulases (Cel7A and Cel7B) acting on suspensions of microcrystalline cellulose (Avicel) at different loads up to 15 g/l. The results showed distinctive differences between the two enzymes. In particular, we found that endo-lytic Cel7B combined very quickly with the substrate and reached the maximal activity within the dead-time of the instrument. Conversely, exo-lytic Cel7A showed a much slower initiation with maximal activity after 5–8 s and a 10-fold lower turnover. We suggest that the instrument may provide an important tool in attempts to elucidate the mechanism of cellulases and other enzymes' action on insoluble substrate.

1. Introduction

Enzyme kinetics is typically studied under steady state conditions. This is based on the assumption that over an extended window of time the concentrations of all enzyme-substrate complexes − and hence the reaction rate − are essentially constant. This approach is appealing because it enables the extraction of some kinetic parameters from relatively simple experiments and analyses, and steady state theory has proven itself extremely useful in the study of countless enzyme catalyzed reactions [1]. The parameters extracted from steady state experiments, k_{cat} and K_M, are often interpreted as the chemical reaction rate constant and a measure of enzyme-substrate affinity, respectively. Strictly, it is only in the case of simple (essentially hypothetical) reactions that this interpretation is correct, while most often the determined parameters are composite quantities that lump together contributions from rate- and equilibrium constants from different steps [2,3]. Consequently, for multi-step reactions, analysis of the transient phase preceding the steady state regime is needed to elucidate the rate constants governing individual (non-rate-limiting) steps. While often challenging to achieve both with respect to experiment and analysis, knowledge of such rate constants makes up an indispensable contribution for the mechanistic understanding of an enzyme system.

A convenient approach to measuring transient kinetics of enzymatic reactions is so-called "rapid mixing" methods [4]. The basic principle entails pumping the enzyme and substrate at high rates into specially devised mixing tees that facilitate homogenization; in some cases within microseconds. Subsequently, the progression of the reaction is monitored either in real time at a fixed location (as in stopped-flow measurements) or by quantifying reaction products at fixed distances from the mixing chamber. In the latter case, the age of the sample can be calculated from volume and flow-rate, while the concentration of products is measured either directly (so-called "continuous flow" measurements) or in quenched samples that are retrieved and analyzed separately ("quenched-flow"). Several excellent accounts of these methods and their application have been published [2,3,5,6]. Instrumentation for rapid mixing techniques with millisecond time resolution is now extensively used to establish rate constants of separate steps in enzyme reactions, but commercial instruments are generally

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unable to accommodate suspensions of insoluble material; particularly so if the suspension (partially) precipitates over the time-scale of the experiment. This technical limitation may well be one of the main reasons that mechanistic understanding of interfacial (heterogeneous) enzyme reactions remains quite undeveloped compared to normal (homogenous) reactions in the bulk. Further studies of heterogeneous mechanisms appear to be well motivated as a multitude of enzyme reactions *in vivo* [6,7] as well as in industrial applications [7,8] indeed occurs at an interface. In light of that, we suggest that the development of better experimental tools to characterize heterogeneous enzyme reactions is a central challenge in current enzymology. Previously, we have tested the use of biosensors functionalized with oxidative enzymes as real time electrochemical reporters of the hydrolysis of cellulose [8–10]. This experimental technique had a response time of a few seconds [10,11], which was sufficient to resolve the transient kinetics for some cellulases. Thus, these enzymes required 10's of seconds to reach (quasi-) steady state when attacking a suspension of cellulose particles. However, inadequate time resolution [11,12] as well as the specificity of the biosensors limits their general applicability. Here, we suggest a different strategy and describe a semi-automated quenched-flow (QF) set-up, which uses peristaltic pumps and a stirred substrate suspension in studies of heterogeneous enzyme reactions. With a dead time under 100 ms, resolution was improved an order of magnitude relative to biosensor assays and two orders of magnitude with respect to batch measurements conventionally used for cellulolytic enzymes. We emphasize that this type of instrument appears relevant for heterogeneous enzyme reactions in general although current examples only include different cellulases.

2. Materials and methods

2.1. Chemicals and enzymes

Unless otherwise stated chemicals were purchased from Sigma-Aldrich (St. Lois, MO). Enzyme assays were carried out in 50 mM acetate buffer, pH 5, supplemented with 0.01% sodium azide. Cel7A and Cel7B were both heterologously expressed in Aspergillus oryzae and purified using the same protocol previously developed for Cel7A [9,10]. Both enzymes showed a single band on an SDS gel. Concentrations of purified stocks were determined by OD_{280} using theoretical extinction coefficients [12,13] of 86.8 $mM^{-1}\,cm^{-1}$ and 71.9 $mM^{-1}\,cm^{-1}$ for Cel7A and Cel7 B respectively. Avicel particles were homogenized using a Turrax homogenizer (IKA ultra-Turrax T8, IKA-Werke GmbH, Staufen, Germany) as described elsewhere [13,14]. Subsequently, the substrate was washed twice in water and once in buffer. The washing procedure was as follows: 50 ml aliquots were incubated in a centrifuge tube with 4 glass beads (3 mm diameter) at a fixed horizontal position for approximately 1 h at 300 rpm in an orbital shaker. The cellulose was sedimented by centrifugation (~1500 RCF) and the supernatant was discarded.

2.2. Quenched-flow system

The quenched-flow (QF) apparatus was constructed according to the schematics in Fig. 1 and installed in an insulated compartment (air-bath), which could be controlled to preset temperatures between approximately 10 °C and 50 °C. Temperature fluctuations in the air-bath over the time scale of the experiment were ± 0.2 °C. All measurements reported here were made at 25 °C. The reagents (enzyme and substrate) were kept in small bottles inside the air-bath. This insured thermal equilibration and, more importantly, allowed magnetic stirring of the substrate suspension, which could hence be kept homogenous. A peristaltic pump (MCP Process ISM 915, Ismatec, IDEX health and science, Wertheim-Mondfeld, Germany), using Masterflex norprene L/S 14 (inner diameter 1.6 mm) tubing (Cole-Parmer Instrument Co., Vernon Hills, Illinois) was also inside the air-bath and delivered the flow of reagents. All other tubing, fittings and PEEK low-pressure mixing tees were purchased from VICI Jour (Schenkon, Switzerland). Connections and delay loops were made with 1/16" outer diameter PEEK tubing connected with flat-bottom flangeless ETFE fittings. Tubing and instrument inner bores were 0.75 mm. The flow path was changed between delay loops using a trapping (ST) flow-path selector valve fitted with an electric actuator (Valco Instruments Co. Inc., Schenkon, Switzerland). The quench solution (1.1 M NaOH) was delivered at 1/10 of the reactants' flow rate by a syringe pump (Fusion 100, Chemyx, Stafford, TX). Samples were collected in a stand-alone fraction collector retrofitted with an Arduino Uno microprocessor (http://www.arduino.cc) for control and interfacing. The entire system was controlled by a virtual instrument written in the graphical programming language LabVIEW (National Instruments, Austin, TX). Hence, experiments through different loop-lengths, collection of quenched samples and rinse cycles between measurements could be pre-programmed and executed automatically. In more practical terms, aliquots of 50–150 ml of enzyme and pre-conditioned substrate were placed in the air-bath for thermal equilibration, and a constant flow was applied. After discarding the first 5 ml, three samples of about 1 ml were collected from the continuous stream of quenched reaction mixture, the ageing loop was switched and the following ~1.5 ml were discarded before collecting the next three samples. Typically, 15–30 samples (corresponding to 5–10 time points) were collected during a continuous run. Subsequently the system was emptied, rinsed and a new run at a different flow rate was carried out.

The rate of reactant flow was frequently calibrated by weighing the output over fixed time intervals. Based on this measured flow rate and the inner bore, the reaction time for each loop could be calculated. To verify this value of the reaction time and the overall performance of the instrument we analyzed the alkaline catalyzed hydrolysis of 2,4-dinitrophenyl acetate (DNPA), which has previously proven well-suited as a control reaction for QF instruments [5]. In the control experiments the reactants were 0.1 mM DNPA and either 25 mM or 100 mM NaOH, while the quenching agent was 4.4 M HCl. Flow rates of respectively 30 ml/min and 60 ml/min were used in the controls.

2.3. Transient kinetics of Cel7A and Cel7B

We analyzed the first 5.7 s of hydrolytic activity of Cel7A and Cel7 B on Avicel by means of QF. Samples were collected in triplicate at 3 different flow rates (60, 20 and 10 ml/min) to achieve a satisfactory time resolution over the full time scale. To investigate the time range beyond 5.7 s we also conducted batch activity measurements according to a slight modification of a previously described protocol [14,15]. Briefly, the reaction was started by adding 3.5 ml enzyme solution to an equal volume of substrate suspension in a stirred, thermostated beaker. Subsets of 800 µl were retrieved after 5, 10, 20 and 40 s for Cel7A and 5, 10, 20 and 30 s for Cel7 B and immediately quenched by mixing into a vial with 80 µl 1.1 M NaOH. Batch measurements were repeated in triplicate. For all (QF and batch) samples residual cellulose was separated by centrifugation (1500 RCF, 5 min). Supernatants were analyzed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS-5000 instrument fitted with a CarboPac PA10 column (Thermo Scientific, Waltham, MA). Cellobiose contents were calculated against a 6-point external standard as described previously [15,16].

3. Results and discussion

3.1. Verification of the quench flow setup

To accommodate insoluble substrates in the QF setup, the flow was delivered by a peristaltic pump. Unlike the conventional use of syringe pumps in QF instruments, this allowed continuous stirring of the substrate suspension. One drawback of peristaltic pumping is the risk that

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