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A graphene screen-printed carbon electrode for real-time measurements of unoccupied active sites in a cellulase

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ABSTRACT

Cellulases hydrolyze cellulose to soluble sugars and this process is utilized in sustainable industries based on lignocellulosic feedstock. Better analytical tools will be necessary to understand basic cellulase mechanisms, and hence deliver rational improvements of the industrial process. In this work we describe a new electrochemical approach to the quantification of the populations of enzyme that are respectively free in the aqueous bulk, adsorbed to the insoluble substrate with an unoccupied active site or threaded with the cellulose strand in the active tunnel. Distinction of these three states appears essential to the identification of the rate-limiting step. The method is based on disposable graphene-modified screen-printed carbon electrodes, and we show how the temporal development in the concentrations of the three enzyme forms can be derived from a combination of the electrochemical data and adsorption measurements. The approach was tested for the cellobiohydrolase Cel7A from *Hypocrea jecorina* acting on microcrystalline cellulose, and it was found that the threaded enzyme form dominates for this system while adsorbed enzyme with an unoccupied active site constitutes less than 5% of the population.

Interest in cellulolytic enzymes has intensified as a result of their role in upcoming sustainable industries producing biofuels and chemicals from lignocellulosic feedstock. Nevertheless, key aspects of cellulase action remain poorly understood, and this is at least in part the result of elusive interactions of the multidomain enzymes and their heterogenous, insoluble substrate. Deeper understanding of these dynamic interactions appears necessary for the identification of rate-limiting steps, and, in turn, the development of better industrial enzymes through rational enzyme engineering [1]. Recent work has suggested that the recognition and association of a segment of the cellulose strand into the active site of the cellulase limit the rate of hydrolysis for crystalline cellulose [2–5]. It follows that improvements of this "threading" step of the cellulase could be a promising strategy for cellulase engineering [2]. However, other results have pointed toward slow dissociation of different types of enzyme–substrate complexes as the rate-limiting step [6–10]. In light of this it appears that better understanding of basic cellulase mechanisms will require both improved analytical techniques, which can detect and quantify the different states of enzyme that occur in the catalytic cycle, and better theoretical tools to rationalize the experimental data. In particular, such information is essential in attempts to model the complex course of events in enzymatic hydrolysis of cellulose. In the current work we suggest one such approach, which allows quantification the three enzyme states (free, associated, and threaded) defined in Fig. 1. Quantitative knowledge of the temporal development of these three states is essential for assessment of the putative rate-limiting steps noted above. This type of information is, of course, primarily relevant for the fundamental understanding of the complex process, but it could also pave the way for knowledge-based solution to some of the countless challenges that must be solved to implement industrial saccharification of biomass, in particular the development of improved enzyme variants. In our discussion of the three states in Fig. 1 we will use the term "associated" for bound enzyme (i.e., enzyme that is removed from solution by filtration or centrifugation) which has a free active site (i.e., the catalytic residues are not blocked by a cellulose molecule). We will use the term "adsorbed" for any enzyme that is not free (i.e., the sum of associated and threaded enzyme in Fig. 1). Experimental concentrations of these states are derived from the combined use of adsorption measurements and a novel continuous electrochemical method based on a graphene-modified screen-printed carbon electrode (SPCE).[2]

Recent progress in experimental methods to determine the different molecular states of adsorbed cellulases includes the use of

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[2] *Abbreviations used:* SPCE, screen-printed carbon electrode; MWCNT, multiwalled carbon nanotube; SWCNT, single-walled carbon nanotube; GPH, graphene; *p*NP, *p*-nitrophenol; *p*NPL, *p*-nitrophenyl β-D-lactopyranoside.

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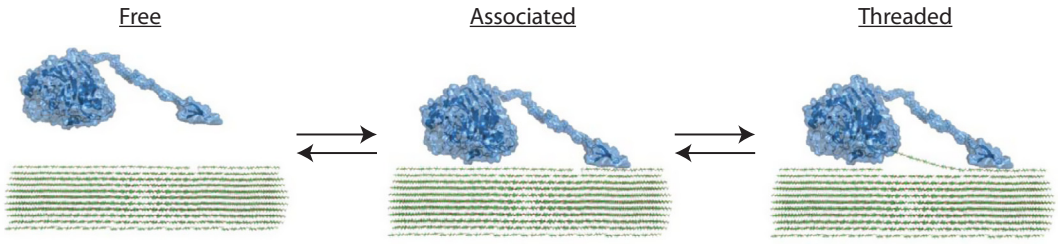

Fig.1. Definition of the three molecular states of a cellulase discussed in this work. The free enzyme in the aqueous bulk becomes adsorbed through binding of the cellulose binding module (CBM). Subsequently the cellulose strand is threaded into the active tunnel to form an activated complex.

surface-sensitive techniques such as quartz crystal microgravimetry [11] and flow ellipsometry [5]. In these works, the separation of the adsorption and hydrolysis processes, and hence threaded and nonthreaded cellulase, relied on measurements with and without glucose added in concentrations that were claimed to inhibit activity but not binding. Other approaches have included batch measurements based on inhibition by a soluble competitor. Kostylev et al. [12], for example, used inhibition by cellotetraose to show that the processive endocellulase Cel9A from *Thermobifida fusca* was predominantly in the associated sate (cf. Fig. 1) when added to a suspension of crystalline cellulose. The distribution between the states in Fig. 1 has also been analyzed using synthetic soluble substrates which release either chromogenic or fluorogenic products such as *p*-nitrophenol and 4-methylumbelliferone [13–15]. If a sample contains both insoluble cellulose and one of these synthetic analogues, the specific inhibition exerted by cellulose on the hydrolysis of the soluble "reporter" substrate allows distinction of threaded enzyme (which is unable to hydrolyze the reporter substrate) and enzyme with an accessible active site (i.e., the sum of free and associated populations in Fig. 1) [8,9]. This approach was used to quantify the threaded concentration of the cellobiohydrolases Cel7A from *Hypocrea jecorina* and Cel7D from *Phanerochaete chrysosporium* on different cellulose substrates [8]. This work used quenching at different time points followed by spectrophotometric quantification. Here, we report an electrochemical approach which allows continuous measurements and about two orders of magnitude higher sensitivity than spectrophotometry.

Experimental

Materials and reagents

Unless otherwise stated, all chemicals were of HPLC grade (>99% purity) and supplied by Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared with 50 mM sodium acetate buffer supplemented with 0.1 M KCl and adjusted to pH 5.0 with 1 M HCl. Stock solutions of *p*-nitrophenyl β-D-lactopyranoside (*p*NPL), *p*-nitrophenol (*p*NP), *p*-aminophenyl β-D-cellobioside (*p*APC, supplied from Santa Cruz Biotechnology (Heidelberg, Germany)), *p*-aminophenyl β-D-lactopyranoside (*p*APL) (prepared by catalytic hydrogenation of 0.5 mM *p*NPL in the standard buffer using 10% Pd on activated charcoal as a catalyst), *p*-aminophenol (*p*AP), 4-methylumbelliferyl β-D-cellobioside (MUC), and 4-methylumbelliferone (MU) were prepared in the buffer and stored in aliquots at −30 °C. Solutions were prepared daily by dilution of thawed stock aliquots. The retaining cellobiohydrolase, Cel7A, from *H. jecorin*a (anamorph: *Trichoderma reesei*) (*Hj*Cel7A) was cloned, expressed, and purified as described elsewhere [16,17].

Electrodes

Screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Oviedo, Spain). Four different types of SCPEs were tested. These were made of carbon either unmodified (DRP-C100), multiwalled carbon nanotube modified (DRP-110CNT), single-walled carbon nanotube modified (DRP-SWCNT), or graphene modified (DRP-110GPH), respectively. The planar SPCEs consists of a conventional three-electrode configuration with a silver pseudo-reference electrode and a carbon counterelectrode printed on a ceramic support with the dimensions of $3.3 \times 1.0 \times 0.05$ cm (length × width × height). The working carbon electrode has a diameter of 4 mm (geometric surface area of 12.57 mm^2). The carbon nanotubes on the MWCNT-SPCE and SWCNT-SPCE were grown by chemical vapor deposition (CVD) process and functionalized with approximately a 5% ratio of carboxylic groups. Further information of the different SPCEs can be found at the supplier's website (http://www.dropsens.com/). The SPCEs were connected to the potentiostats through a specific connector from DropSens. In addition to the screen-printed electrodes we also tested a number of normal disk-type carbon-based electrodes including glassy carbon, carbon paste, and plastic formed carbon as well as basal- and edge-plane pyrolytic graphite.

Electrochemical measurements

A VersaSTAT 3F from Princeton Applied Research (Tennessee, US) or a HECS 972 potentiostat from Husou Seisakusyo Co. (Kawasaki, Japan) was used for cyclic voltammetry. The HECS 972 were connected to a computer via GPIB-interface and controlled with a home-written C++ program. Constant-potential amperometry was performed with a 1112 potentiostat from Husou Seisakusyo Co., which was connected to a computer via a Agilent 34401A DMM and a AD/DA converter with a data acquisition program written in LabVIEW 2012 (National Instruments, Austin, USA). Electrochemical measurements were performed in 10 ml solution in a water-jacketed glass cell connected to a water bath. All applied potentials are specified with respect to the internal Ag-pseudo reference electrode of the SPCEs (55.5 mV vs SCE (Bioanalytical Systems, UK) in the buffer). Prior to all measurement the SPCEs were pretreated by cycling the potential 10 times between +0.1 and +1.1 V with a scan rate of 50 mV s^{-1} in the buffer. The SPCEs were electrochemically characterized by cyclic voltammetry in 1 mM aqueous solution of the outer-sphere one-electron redox probe potassium ferricyanide in 0.1 M KCl. Cyclic voltamograms were recorded by cycling the potential between +0.1 and +1.1 V with a scan rate of 50 mV s^{-1} in 1 mM *p*NP solution. In the amperometric measurements the solution was stirred by a magnetic bar (20×3 mm) at 500 rpm.

Electrochemical enzyme assay

Calibrant (*p*NP, *p*AP or MU solution) or enzyme solution was delivered to the reaction vessel from a 100-μl syringe (SGE Analytical Science, Melbourne, Victoria, Australia) mounted in a syringe pump (Fusion 100, Chemyx, Stafford, TX) through 0.125-mm i.d. PEEK tubing. Calibration experiments were conducted by