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Review

Single-enzyme kinetics with fluorogenic substrates: lessons learnt and future directions

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ABSTRACT

Single-molecule fluorescence techniques have developed into powerful tools for studying the kinetics of biological reactions at the single-molecule level. Using fluorogenic substrates, enzymatic reactions can be observed in real-time with single-turnover resolution. The turnover sequence contains all kinetic information, giving access to reaction substeps and dynamic processes such as fluctuations in the reaction rate. Despite their clearly proven potential, the accuracy of current measurements is limited by the availability of substrates with 1:1 stoichiometry and the signal-to-noise ratio of the measurement. In this review we summarize the state-of-the-art and discuss these limitations using experiments performed with α-chymotrypsin as an example. We are further providing an overview of recent efforts aimed at the improvement of fluorogenic substrates and the development of new detection schemes. These detection schemes utilize nanophotonic structures such as zero mode waveguides or nanoantennas. Nanophotonic approaches reduce the size of the effective detection volume and are a powerful strategy to increase the signal-to-noise ratio. We believe that a combination of improved substrates and novel detection schemes will pave the way for performing accurate single-enzyme experiments in biologically relevant conditions.

1. Introduction

Enzymes are dynamic molecules with the ability of catalyzing biochemical reactions with high rate accelerations and specificity. The origin of this dynamic nature is a multidimensional energy landscape that does not only define the 3D structure but also the catalytic function as well as regulation processes. Over the past few decades, single-molecule techniques, especially single-molecule fluorescence spectroscopy (SMFS) and microscopy, have evolved into powerful tools for studying enzymatic reactions. Advances in measurement technology and the development of fluorescence-based reporter systems [1–4] have improved the sensitivity and the time resolution to an extent such that dynamic processes during the catalytic reaction can be monitored in real-time, directly giving access to the sequence of events. Temporal fluctuations of enzyme behavior as well as heterogeneities between different enzymes in the population can be determined directly in single-molecule experiments. This information is inaccessible in ensemble measurements.

Conformational changes can, for example, be monitored utilizing Förster Resonance Energy Transfer (FRET). FRET is a sensitive spectroscopic ruler that reports on the distance between two fluorophores in close proximity. The energy transfer efficiency is a direct readout of the fluorophore distance and allows for detecting distance changes in the range of several nanometers [5–12]. At the single-molecule level (smFRET), when the donor and acceptor fluorophores are attached to specific positions on an enzyme, the energy transfer efficiency (E_{FRET}) provides a direct readout of conformational changes. Ha et al. [5] were the first to utilize this principle for monitoring the conformational dynamics of staphylococcal nuclease. Fluctuations in E_{FRET} occurred on the millisecond to second timescale, which is characteristic for functionally relevant conformational changes. The strength of this detection scheme is at the same time one of its biggest weaknesses. In order to obtain meaningful information about conformational motions, the locations of the donor and acceptor fluorophores need to be known exactly. This requires prior knowledge of the enzyme structure and an advanced specific labeling procedure [13–15].

smFRET can also be used for following reaction substeps and intermediate states of enzymes containing an optically active cofactor, such as in many oxidoreductases. The enzymatic turnover cycle can be observed directly when the cofactor cycles between an oxidized and a reduced state with different spectral properties

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[16–21]. A cofactor that absorbs light in only one of its oxidation states can be used as a FRET acceptor. Labeling the enzyme with a donor fluorophore in close proximity to the cofactor will consequently lead to changes in the donor fluorescence intensity as a function of the oxidation state of the cofactor. FRET labeling is not required when the cofactor itself is fluorescent in its oxidized state [16–18]. In this case the redox cycling of the cofactor can be followed directly.

One of the most intriguing discoveries of single-molecule enzymology has been the observation of temporal fluctuations in the activity of enzymes, a phenomenon named dynamic disorder [16,22–25]. These temporal fluctuations in the rate-limiting step have been the basis for the 'fluctuating enzyme model' that was developed to explain these observations [23]. This model relates fluctuating rate constants with conformational changes, assuming that each conformation has its own specific activity. A reliable identification of dynamic disorder requires the detection of a large number of individual enzymatic turnovers. Sufficient statistics is required to accurately detect the underlying dynamic processes that may span a large range of timescales. The acquisition of the required data is only possible with a suitable reporter system that allows for long measurement times. The reporter systems mentioned above each possess certain technical limitations preventing such long measurement times and consequently the accurate analysis of dynamic heterogeneities in the catalytic activity. smFRET does 'only' provide information about conformational changes but does not directly detect enzymatic turnovers. Cofactor-based detection schemes are naturally limited to enzymes containing an optically active cofactor. For both detection schemes, the measurement is based on following the same fluorophore over the time course of the experiment. The measurement is consequently terminated by photobleaching of the fluorophore, limiting the data acquisition time for one single enzyme to only a few seconds.

Fluorogenic substrates, which are converted by the enzyme into fluorescent product molecules, are in principle the only reporter systems that permit sufficiently long measurements. Fluorogenic substrates are the ideal system for studying enzyme kinetics as a new fluorophore is produced during every enzymatic turnover [22–24,26–31]. In this paper, we review the current state of single-enzyme experiments focusing on fluorogenic substrates as reporter systems. We will first describe the technological requirements and the basic experimental setup used for performing these experiments. In the following, we will describe a recent single-molecule study of α-chymotrypsin as an example to highlight the unique information that can be obtained in such an experiment. Using this same example, the current limitations of the substrate design and the measurement setup will be discussed together with recent developments aimed at overcoming these limitations. These include improved fluorogenic substrates as well as single-molecule detection schemes. These technological advances have the potential to improve the signal-to-noise ratio of the measurement and will ultimately help to perform single-enzyme experiments under biologically relevant conditions while at the same time allowing for a more accurate kinetic analysis.

2. Single-turnover detection

The ideal single-turnover measurement requires the direct observation of every enzymatic turnover with a signal-to-noise ratio (SNR) high enough for accurate data interpretation (Fig. 1a). Fluorogenic substrates provide this direct readout of individual enzymatic turnovers. They consist of a fluorophore linked to a functional group that is recognized by the enzyme. This functional group alters the photochemistry of the fluorophore, making it non-emissive. After enzymatic cleavage of the functional group, the fluorophore recovers its fluorescence. To be able to follow the sequence of turnovers, the enzyme needs to be immobilized on the surface of a glass cover slip. The enzyme itself is also fluorescently labeled so that every individual enzyme can be located on the surface. Upon the addition of substrate, the fluorescence signal at the position of one enzyme is recorded. Enzyme activity results in the formation of fluorescent product molecules at this specific position on the surface. Using a confocal microscope, every product molecule is detected as a fluorescence burst above the background

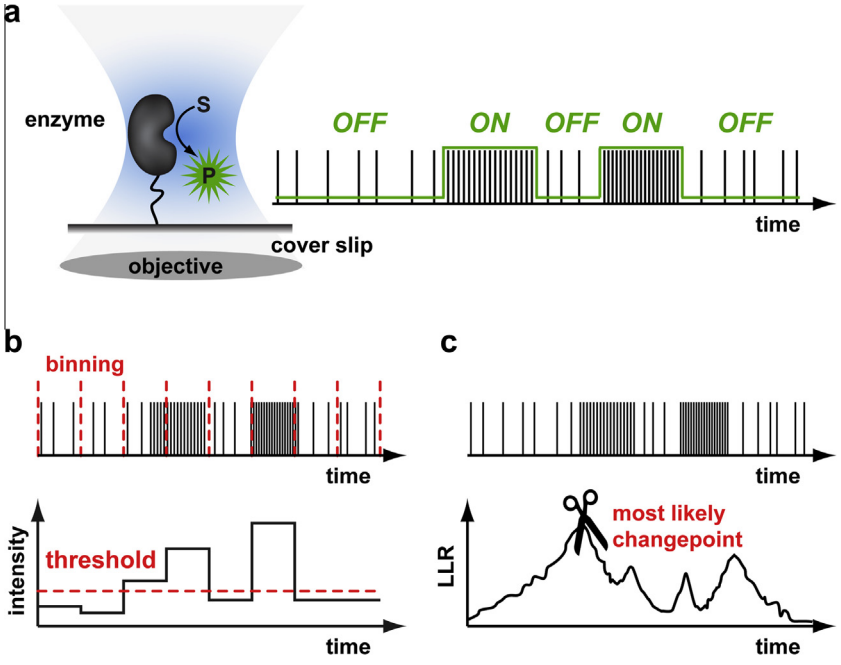

Fig. 1. Single-turnover detection with fluorogenic substrates. (a) The laser of a confocal microscope is focused onto the position of an immobilized enzyme on a glass cover slip. Every fluorophore produced by the enzyme is recorded in real-time with single-photon sensitivity yielding the arrival time ("macro-time") of every individual photon. Threshold analysis (b) or change point analysis (c) are used to assign the ON- and OFF-times to the photon arrival time trace.