CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 44, Issue 9, September 2016
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Anal Chem, 2016, 44(9), 1437–1446.

REVIEW

Research Progresses in Single Molecule Enzymology

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Abstract: The advent of single molecule imaging technologies in 1990s made the dynamics of single molecule enzyme catalysis be successfully probed in real time *in vitro*. Ever since then, single molecule enzymology entered the golden age of rapid developing. Individual features of single enzyme molecule hidden in the overall average were discovered, and many new catalytic mechanisms were proposed. Single molecule enzymology sheds light on the dynamic interactions between enzyme and substrate or product, deepening the understanding of biochemical reactions.

Key Words: Single molecule; Enzyme; Ribozyme; Fluorescence resonance energy transfer; Fluorescence microscopy; Review

1 Introduction

As an important constituent part of biology, enzyme could catalyze a series of biochemical reactions, making it an indispensable element for organisms to maintain normal physiological requirements. Although enzyme has been studied for a long time, new questions in understanding how enzyme functions in real time arise. How does individual enzyme dynamically change during the catalytic process? Is there any difference among individuals of enzyme? How does enzyme interact with its substrate? Nevertheless, traditional measurements often recorded the overall average, and the results represented overall similarities of countless molecules, and could not reflect the individual characteristics of single enzyme. It is an impossible mission for typical traditional ensemble measurements to probe specific features of single enzyme functions. The development of technology has expanded the horizons of thinking as well as exploring. In the early 1990s, researchers set out to participate in the development of single molecule imaging techniques at room temperature[1–7]. The continuing improvements of imaging, detection as well as single-molecule manipulation offered suitable opportunities for the observation of biological macromolecules functions in single molecule level at that time. The application of single molecule fluorescent microscopy provided an excellent avenue to monitor the dynamic motions of individual molecule, and addressed the dynamic features of enzyme from deeper depth. The present single molecule study uncovered the dynamic feature of enzyme-activity of single enzyme fluctuates during catalysis. Afterwards, this phenomenon has brought new concerns that what cause enzyme fluctuate? As known to all, enzyme is constituted of amino acids chains (polypeptide chain) and only when the polypeptide chain folds into the specific configuration, could enzyme functions normally as biocatalyst to generate product. However, in the presence of thermal motion, polypeptide chains have to fluctuate to maintain the specific configuration, and the fluctuation of polypeptide chains inevitably induces conformational fluctuation of enzyme[8]. That is why enzyme could fluctuate in different conformations, each with specific activity. Meanwhile, the energy landscape determines the thermodynamically and kinetically accessible conformations, their relative ratios and interconversion rates. Therefore, enzyme turns over many times before converting to a low active state, which gives the explanation for memory effect.

Studying enzyme catalysis at single molecule level by using fluorescence microcopy becomes an important research field. The general study procedures are shown as follows. Firstly,

Received 18 March 2016; accepted 18 April 2016
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This work was supported by the National Natural Science Foundation of China (Nos. 21227804, 21373260, 31371015), the Youth Innovation Promotion Association, Chinese Academy of Sciences, and the Open Project of State Key Laboratory of Oral Diseases of China (No. SKLOD2015OF05).

DOI: 10.1016/S1872-2040(16)60957-X

enzymes are loosely fixed onto the quartz or glass interface. Secondly, enzyme is immersed in the reaction buffer which contains the substrate, then the enzyme starts to catalyze the substrate to generate product. During the catalytic process, the interactions between enzyme and substrate or product would lead to the fluorescent change which could be recorded by fluorescent microscopy. Thirdly, fluorescent intensity trajectories of single enzyme are obtained and analyzed to build suitable catalytic model. The study is based on fluorescent change. However, owing to the limited experimental conditions, several rules should be considered to carry out a single molecule study: (1) the fluorescent active state of enzyme can be changed during the reaction; (2) the enzyme could catalyze fluorogenic substrate to generate fluorescent product; (3) the substrate could be easily labeled with flurophore and its fluorescent state may change after the reaction; (4) the enzyme or enzyme and substrate could be labeled with fluorescence resonance energy transfer (FRET) pairs to study the conformational change; (5) the intensity of fluorophore could be dramatically changed due to conformational change of enzyme. Researchers could independently record the fluorescent signals generated by the catalytic process of enzymes through single molecule fluorescent microscopy, then build mathematical catalytic model through statistical analysis of intensity trajectories. Therefore, single molecule enzymology could obtain the dynamical fluctuation of individual enzyme and provide statistical and theoretical support for understanding a specific catalytic system.

In this review, we make a brief introduction of research progress in single molecule study on enzyme and ribozyme. We hope it will help the readers make a better understanding of single molecule enzymology.

2 Research progresses in single molecule enzymology

2.1 Research progress in single molecule protein enzyme

The advent of DNA double-helix structure model has opened a new chapter of interpreting structures of biological macromolecules. Essential to all life, enzyme is an indispensable element for organisms to maintain normal physiological functions. Although crystal structures of different types of enzyme have been resolved using X-ray crystallography and other physical techniques, we are still confused with some problems. How does enzyme work in real time during catalysis? What is the relationship between structure and function of enzyme? While through observation of enzyme catalysis from single molecule perspective, these questions have been gradually uncovered. The pioneer work of single molecule study of enzyme was conducted by Lu *et al*[9] through real-time observation of catalytic process of cholesterol oxidase (COx) in 1998. As shown in Fig.1A, the active center of COx involves a flavin adenine dinucleotide (FAD), the oxidized form of FAD is naturally fluorescent while the reduced form is not fluorescent active. When COx catalyzing its substrate, FAD is reduced to the reduced form ($FADH_2$) followed by the re-oxidization to FAD, and then a new catalytic cycle starts. The emission intensity is recorded in real time through an inverted fluorescence microscopy. Figure 1B shows a typical intensity trajectory of single COx. It is obvious that the fluorescent intensity stochastically fluctuate, and each on-off represents a complete catalytic turnover. The authors separated the whole reaction into two parts to simplify the analytic procedures, and analyzed a series of on-time and off-time using mathematics. The authors made product generation process rate limiting using a cholesterol derivative to evaluate the rate constant variance of individual enzyme. The result is illustrated in Fig.1C. The distribution of on-time was monotonic exponential decay and the distribution of rate constant among individuals of enzyme was broad. This discovery indicated that static disorder was present in enzyme catalysis[10]. Previous bulk measurements proposed that enzyme catalysis was a Markovian process, the catalytic rate was constant and there was no cross influence between adjacent two reactions. However, as shown in Fig.1D, there was an obvious diagonal feature which indicates the memory effect between the adjacent on-time pair. Moreover, when quantitatively analyzing the difference using autocorrelation function (r), r(m) decayed when the two-turnover pair was separated by increasing numbers of turnovers (Fig.1E). This discovery indicated that the reaction velocity and configuration of enzyme fluctuated during catalysis, and verified that there was no specific rate constant for enzyme from single molecule perspective. In the subsequent study, Yang *et al*[11] again confirmed that the conformational fluctuation of enzyme was a common phenomenon when using electron transfer as probe to correspond to distance variances. In general, probing conformational change of enzyme is a difficult task for ensemble averaging. However, individual features of single enzyme could be recorded and analyzed to reveal new insights in catalytic mechanism through single molecule techniques. The experimental designs and analytical methods developed by this single molecule assay made solid foundations for researching on different types of enzyme.

In addition to the above mentioned COx whose fluorescent active state could be altered during catalysis, there was another kind of enzyme which catalyzed fluorogenic substrates to generate fluorescent products, this feature made it suitable candidate for single molecule study. Different from previous kind of enzyme, the second type had no fluorescent active state, and the observed fluorescent transformation was caused by the fluorophore generation reaction catalyzed by enzyme. It was noted that the substrate-product could not