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Online kinetic studies on intermediates of laccase-catalyzed reaction in reversed micelle

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Abstract

Using water/AOT/*n*-octane reversed micelle as the medium, the optical signal of the reactive intermediate of laccase-catalyzed oxidation of *o*-phenylenediamine, which was indetectable in aqueous solutions, was successfully captured. Thus online kinetic studies of the intermediate were accomplished. Two-way kinetic spectral data were acquired with stopped-flow technique. By resolving the data with global analysis software, both the kinetic curves and the absorption spectra of the components involved in the reaction process were simultaneously obtained. The whole reaction in the reversed micelle was proved to be composed of two successive steps, an enzymatic generation of the intermediate and a following nonenzymatic decay of the intermediate. A consecutive first-order kinetic model of the whole reaction was confirmed. The influences of microenvironmental factors of the medium (such as the pH value of the water pool and the water/AOT ratio) on the detection of the intermediate were also investigated.

Keywords: Reversed micelles; Reactive intermediates; Online kinetic studies; Stopped flow; Laccase

1. Introduction

Since it has been well established that many chemical and biochemical reactions, both in vivo and in vitro, are enzyme-based, the great importance of studying enzymatic reactions has now been fully recognized. The understanding of the mechanism of these reactions is considered to be especially significant. As is known, enzymatic intermediates always contain very essential and valuable information about processes of enzymatic reactions. Thus they have long been the focus of research in this field [1–3]. However, researchers have encountered numerous difficulties in obtaining and analyzing information on highly reactive intermediates, mainly due to features such as the rapid reaction rate, the extremely short lifetime, and the very weak signal. Great effort has been made to overcome these difficulties [4–6], which can be classified into two types. One is to stabilize the enzymatic intermediate and to enhance its detectable signal by optimizing reaction conditions (called chemical approaches); the other is to reform experimental methods and to improve the capability of detectors in order to "capture" the electrical or optical signal of the intermediate that is unacquirable with common methods or apparatus (called physical approaches). Since the development of new experimental techniques and instruments is relatively slower and more difficult, more attention has been paid to finding new ways to stabilize and enhance the signal of enzymatic intermediates. Many of these methods, however, often need specific reagents with complicated operations or need rigorous experimental conditions such as extremely low temperature. Some offline methods are used to detect the enzymatic intermediate by turning it into another long-life species [7], but they are not able to trace the actual dynamic process that is essential to understanding the reaction mechanism. Therefore, it is still necessary to find new methods for studying enzymatic intermediates, especially for the purpose of online analysis of the enzymatic reaction process.

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Reversed micelles are a class of molecular aggregates formed by kinds of amphiphiles assembling into microemulsion droplets in organic solvents in the presence of water [8]. The minute quantities of water solubilized in the inner core always exhibit typical solvation properties [9]. Hence they are highly interesting from the physicochemical and biochemical viewpoint [10–12]. In our previous work, the ability of reversed micelles to act as solubilizers and sensitizers in quantitative analysis was proved, which was mainly due to the special solubilizing power of the inner water [13]. As is well known, physical and chemical properties of the inner water are distinctly different from that of bulk water, but similar to that of biological interfacial water in several aspects. What is more, the multiphase system is thermodynamically stable and optically transparent, which makes it directly and easily measurable with photometric methods. Therefore, we consider that reversed micelles might be competent to capture optical signals of enzymatic intermediates for online kinetic studies of enzymatic reactions.

In this work, laccase-catalyzed oxidation of *o*-phenylenediamine (OPDA) was studied on line in water/AOT/*n*-octane reversed micelle. Laccase (EC1.10.3.2) is a kind of phenoloxidase widely distributed in many plants and numerous fungi. Many reports have been made concerning the purification, characterization, and activity determination of laccase [14], but the mechanism of laccase-catalyzed reactions is still in argumentation because of its complexity. Researchers have tried hard to get information on laccase-based intermediates [15]. But the reported methods, as mentioned above, are rather inconvenient and extremely costly. In the present work, using water/AOT/*n*-octane reversed micelle as the medium, a very clear and strong spectral signal of the intermediate was observed, which was entirely indetectable in aqueous solutions. A stopped-flow technique was applied in the work, as it has been proved to be powerful and effective in online kinetic studies [16–18]. Stopped-flow kinetic analysis has played a very important role in studying enzymatic intermediates [19,20]. Combined with a multiple-channel detector such as a photodiode array (PDA), it always provides two-way kinetic data for analysis. The results derived from such multidimensional data are highly robust and reliable since they have made full use of the complete information on the reaction process [21,22]. In this work, two-way kinetic spectral data containing information on both the time dimension and the wavelength dimension were obtained and resolved. Kinetic parameters of the enzymatic intermediate were calculated and a kinetic model of the laccase-catalyzed oxidation of OPDA was thus built.

2. Materials and methods

2.1. Materials

Sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) was purchased from Fluka and dried with P_2O_5. *n*-Octane and *o*-phenylenediamine (OPDA) were purchased from the Shanghai Reagent Factory and dehydrated through distillation or sublimation before use. Laccase (extracted from Chinese sumac) was kindly donated by Professor Yu-ming Du's group at Wuhan University, and 1 mg ml^{-1} storing solution was prepared in water. Na_2HPO_4–HAc buffer was used to adjust the pH value of the water pool as well as the W value (water vs surfactant in mol) of the reversed micelle. All the chemicals used were of analytical reagent grade or better and distilled deionized water was used throughout.

2.2. Methods

AOT was resolved in *n*-octane to prepare a 0.2 mol l^{-1} solution. A very small quantity of aqueous solution of substrate (OPDA) or laccase was added in the AOT/*n*-octane system; thus reversed micelle solutions were prepared. All solutions were then adjusted to the same W value (water/surfactant in mol) with Na_2HPO_4–HAc buffer. The reversed micelle solutions of substrate and enzyme were pumped into a 20-μl flowing cell through separate streams. Kinetic spectrophotometric measurements were performed with an SX18MV-R stopped-flow reaction analyzer (Applied Photophysics, UK) with the temperature being controlled by a TB-85 thermo bath (Shimadzu). The two-way kinetic spectra data were recorded with a PDA detector (Applied Photophysics, UK). Data were transferred into ASCII format and processed with Pro-Kineticist II global analysis software (accompanied by the stopped-flow analyzer). The software, as described previously [23,24], allows researchers to globally analyze a complete data set according to a proposed reaction scheme, providing a best-fit parameter set that will accommodate all kinetic behavior across the entire measured wavelength range.

3. Results and discussion

3.1. Capturing the intermediate information in reversed micelle

The two-way kinetic spectra of the reaction process were recorded both in aqueous solution and in reversed micelles. As can be seen in Fig. 1, the illustrations intuitively proved the presence of the optical signal of laccase-based intermediate in the reversed micelle. There had an absorbing band with a peak located at 425 nm, both in aqueous solution (Fig. 1a) and in reversed micelles (Fig. 1b), which came from the final product, 2,3-diaminophenazine (DAP) [25]. But a noticeable difference was that an absorption peak located around 364 nm was clearly seen in the reversed micelle, whereas it did not appear in the aqueous solution. As regards the temporal dimension, the intensity of the absorption peak increased first and decreased subsequently, apparently in accord with the kinetic characteristics of reactive intermediates.