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Inactivation studies on native and silica gel non-homogeneous immobilized chloroperoxidase

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Abstract

A pH stability analysis was made, in the presence of *tert*-butyl hydroperoxide, of both immobilized and native chloroperoxidase obtained from *Caldariomyces fumago* and the inactivation constants (j_1) evaluated. The native enzyme displays a uni-exponential decay, whereas for the immobilized enzyme a three exponential equation describes the time dependent enzyme inactivation.

For immobilized enzyme, three-exponential equation describes the enzyme time-course inactivation. The obtained inactivation constants (j_3 and K_3) showed an increase in the stability of a fraction of the immobilized enzyme. This is probably due to a decrease of the affinity of the enzyme for the oxidant and not to a decrease in j_3 values.

Keywords: Suicide substrate; Kinetic parameters; Enzyme inactivation; Chloroperoxidase; Time course; Immobilized-enzyme

1. Introduction

Chloroperoxidase from *Caldariomyces fumago* is a versatile and unusual heme-peroxidase. In vitro, chloroperoxidase shows halogenase-, peroxidase-, catalase-activity, and in particular some chloroperoxidase-catalyzed reactions appear to be mediated by cytochrome P450-like mechanisms in which the oxygen of the ferryl ($Fe^{IV}=O$) species, which derives from H_2O_2, is transferred to the substrate [1]. The mammalian cytochrome P450 monooxygenases catalyze the metabolism of a wide variety of endogenous and exogenous compounds, including steroids, therapeutic drugs, and carcinogens [2]. In some cases, the formation of a reactive intermediate by P450 may also lead to the inactivation of the enzyme. P450 substrates, which are metabolized to reactive intermediates that inactivate the enzyme, are classified as mechanism-based inactivators [3]. Mechanism-based inactivation of P450 enzymes involves metabolic activation of the substrate followed by binding of a reactive intermediate to either the heme- or the apo-protein to render the enzyme inactive [4]. So, in several works there are cytochrome P450 time- and concentration-dependent inactivation studies that reflect the suicide nature of several P450 substrates.

The progress curve for the irreversible product accumulation will be described by integrated Michaelis–Menten equation [5], in which the dependence of [S] on time is implicit. If the substrate is a suicide one, the enzyme suffers a loss of activity during the incubation. Waley [6,7] proposed implicit integrated equations which describe either the consumption of substrate or the loss of enzyme activity. From mechanism discussed by Waley it was successively derived [8] explicit time-course equations for the accumulation of the product assuming that the substrate concentration remains approximately constant during the period considered. That condition can be achieved experimentally using a suitable value of the enzyme concentration. The experimental design has been applied to the kinetic study of the inactivation of tyrosinase by various suicide substrates [9,10].

Duggleby has made an important theoretical contribution to the problem of enzyme systems in which one or more of the enzyme forms are unstable [11]. The approach is based upon measurement of progress curves of the substrate utilisation. Duggleby suggests a graphical method, the *J* plot, for the

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determination of the inactivation rate constants from the final substrate concentration.

Kinetic analysis has been made for the case in which the free enzyme, or the enzyme–substrate complex, or both, are unstable, either spontaneously or as a result of the addition of a suicide substrate. The explicit time-course equations of all of the species involved have been derived [12–14] assuming that the substrate concentration remains approximately constant during the period considered. That condition can be achieved experimentally using a suitable value of the enzyme concentration. However, it is well known that when an enzyme able to catalyse an irreversible two substrates/two products reaction is incubated with only one substrate, the total concentration of the substrate can be considered as a constant. In our case the only substrate used is the oxidant whose concentration will be constant and we study the enzyme inactivation.

Interest in these systems stems not only from their obvious biological importance but also from a desire to harness their synthetic potential. Because alkane hydroxylation and olefin epoxidation are important industrial reactions, engineered immobilized enzymes that could efficiently activate and transfer oxygen would be of considerable economic value [15]. In comparison with their native form, immobilized enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of continuous processes and repetitive enzyme use. However, in the covalent immobilisation, an enzyme suffers a loss of homogeneity: each appeared enzyme population type shows different half-life after incubation in a simple pH buffer solution. Moreover, a suicide substrate is present, the problems enormously increase.

The aim of this paper is to derive the time-course equation of the oxidant inactivated species under assumptions mentioned in theory and by using these kinetic analyses to study the inactivation of the silica-immobilized chloroperoxidase in the presence of the oxidant *tert*-butyl hydroperoxide.

2. Materials and methods

2.1. Experimental

2.1.1. Materials

2.1.1.1. Reagents. Silica gel (5 μm, specific surface of 340 m^2/g) was from Alltech. (3-Glycidyloxypropyl)trimethoxysilane was supplied by Fluka. Chloroperoxidase (CPO) from *C. fumago* (chloride hydrogen-peroxide oxidoreductase [EC 1.11.1.10]) and *tert*-butyl hydroperoxide were supplied by Fluka and were used without further purification. Monochlorodimedone was a Sigma Chemical Co. product and was used without further purification. All other reagents were commercial products of analytical grade. UV and visible spectrophotometric measurements were performed at room temperature on Varian CARY 04-E spectrophotometer interfaced to a personal computer.

2.1.1.2. Preparation of immobilized chloroperoxidase. Epoxide derivatized silica gel was prepared according to the methods reported [16]. Four milliliters of 0.05 M potassium phosphate buffer solution, pH 6, containing ammonium sulphate 2 M and 250 μl of CPO suspension (12.6 mg/ml) were added to 0.5 g of functionalised silica gel. The mixture was stirred at room temperature for 2 h. The solid with the immobilized enzyme was then recovered through centrifugation and the UV spectrum of the supernatant was recorded at 400 nm. The recovered solid was washed with the same buffer (0.05 M, pH 6) until the disappearing of the maximum at 400 nm in the UV spectrum ($\varepsilon_{400} = 91\ mM^{-1}\ cm^{-1}$). The amount of enzyme bound to the support (2.71 mg CPO/g of silica gel) was calculated from the UV absorbance by subtracting the absorbance of the supernatant plus washings from the initial absorbance of the enzyme solution. The experiment was carried out in duplicate. The SiO_2–CPO preparations were stored at −20 °C until use.

2.1.2. Methods

2.1.2.1. Native chloroperoxidase stability studies. The stability of chloroperoxidase in the presence or the absence of the oxidant, *tert*-BuOOH, was investigated. Ten microlitres of Fluka commercial enzyme were pre-incubated in 10 ml of 0.1 M potassium phosphate buffers of five different pH values (pH 2.75–7). The pre-incubations were performed in triplicates. Ten microlitres of Fluka commercial enzyme were pre-incubated with different concentrations of the oxidant (0.1–7.3 mM) in 10 ml of 0.1 M potassium phosphate buffer (pH 6). The pre-incubations were performed in duplicates with three data point per concentration.

During all pre-incubations, the tubes were shaken at 200 rpm by using an orbital shaker.

2.1.2.2. Silica–chloroperoxidase stability studies. The stability of immobilized-chloroperoxidase in the presence or the absence of the oxidant *tert*-BuOOH was investigated. Ten milligrams of immobilized enzyme were pre-incubated in 10 ml of potassium phosphate buffers (0.1 M) at five different pH values (pH 2.75–7). The pre-incubations were performed in triplicates. Ten milligrams of immobilized enzyme were pre-incubated with different concentrations of the oxidant (0.1–7 mM) in 10 ml of 0.1 M potassium phosphate buffer (pH 6.00). The pre-incubations were performed in duplicates with three data point per concentration.

During all pre-incubations, the tubes were shaken at 200 rev/min by using an orbital shaker.

2.1.2.3. Spectrophotometric assay. A fixed amount (200 μl) of pre-incubation solutions (native, 2.2 U/ml; immobilized, 2 mg/ml) was taken (from 0 to 10^6 s) and added to a standard reaction mixture, consisting of potassium chloride (0.11 mmol), *tert*-butyl hydroperoxide (0.2 mmol), monochlorodimedone (MCD, 0.27 μmol) in 0.1 M potassium phosphate buffer (pH 2.75) in a total volume of 5.4 ml. The reaction was followed by monitoring the decrease in absorbance at 278 nm due to the conversion of monochlorodimedone ($\varepsilon_{278} = 1.22 \times 10^4$) to dichlorodimedone (DCD, $\varepsilon_{278} = 1.6 \times 10^2$) [17].