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Kinetic and stability studies on the chloroperoxidase complexes in presence of *tert*-butyl hydroperoxide

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Abstract

The inactivation of native chloroperoxidase (CPO) from *Caldariomyces fumago* in the presence of *tert*-butyl hydroperoxide (*tert*-BuOOH) was investigated. A kinetic analysis was made and the inactivation constants (V_3 and K_3) were evaluated. In prolonged times, uni-exponential equation describes the enzyme time course inactivation. A method based on the rate of inactivation of the enzyme in the presence of the inactivating molecule *tert*-BuOOH was also performed. A second group of inactivation constants (j_3 and K) was obtained, which is sufficiently close to the first two, thus verifying that the decreasing of enzyme absorbance corresponds to the decay of activity.

Keywords: Suicide substrate; Kinetic parameters; Enzyme inactivation; Chloroperoxidase; Cytochrome P450; Time course

1. Introduction

Chloroperoxidase (CPO) from *Caldariomyces fumago* is the most versatile and unusual heme-peroxidase. In vitro, chloroperoxidase shows halogenase-, peroxidase-, catalase- and cytochrome P450-like activities [1]. CPO and cytochrome P450 play critical roles in physiological processes such as xenobiotic metabolism [2], neurological development [3], blood pressure control [4,5] and immune defense [6]. These thiolate-ligated enzymes are unique among heme proteins in that they catalyze the insertion of an oxygen atom, derived either from molecular oxygen or peroxide, into a variety of organic substrates, often with high degrees of regio- and stereoselectivity. Interest in these systems stems not only from their obvious biological importance but also from a desire to harness their synthetic potential. Most often, P450-mediated biotransformation results in polar metabolites that are inactive, both therapeutically and chemically. However, compounds may be metabolically activated to reactive species that can covalently bind to intracellular macromolecules, potentially causing various drug interactions and/or drug toxicities. In addition, these electrophilic metabolites may covalently bind to the P450 enzyme itself, causing a mechanism-based time-dependent loss of enzyme activity [7]. 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 [8]. So, in several works, there are cytochrome P450 time- and concentration-dependent inactivation studies that reflect the suicide nature of several P450 substrate.

The progress curve for the irreversible product accumulation in enzymatic reactions is described by integrated Michaelis–Menten equation [9] 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 [10,11] proposed implicit integrated equations which

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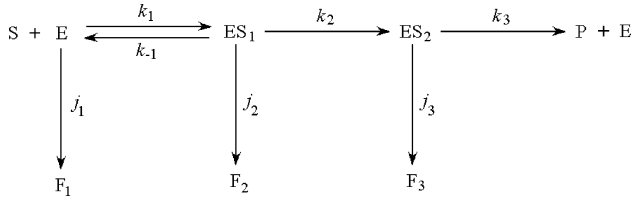
describe either the consumption of substrate or the loss of enzyme activity. From the mechanism discussed by Waley, explicit time course equations for the accumulation of the product were successively derived [12] assuming that the substrate concentration remains almost constant during the time considered. This condition can be achieved experimentally by using a suitable value of the enzyme concentration. This experimental design has been applied to the kinetic study of the inactivation of tyrosinase by different suicide substrates [13,14]. Duggleby [15] made an important theoretical contribution to the problem of enzyme systems in which one or more of the enzyme forms are unstable. The approach is based upon measurement of progress curves of the substrate utilization. Duggleby suggested a graphical method, the *J* plot, for the determination of the inactivation rate constants from the final substrate concentration. A kinetic analysis has been made for the case in which either the free enzyme or the two types of enzyme–substrate complexes are unstable, spontaneously or as a result of the addition of an oxidant, respectively; the explicit time course equations of all of the species involved have been derived [16–18] assuming that, during the time considered, the oxidant concentration remains approximately constant. In the usual enzymatic cycle of peroxidases, the native enzyme reacts with an oxidant to form intermediate compound I with two oxidising equivalents above ferric state; compound I is then reconverted to the native form via one to two electron steps in which two reducing substrate molecules are oxidized into radicals [19]. But the peroxidation of chloride catalyzed by the chloroperoxidases differs from the above mechanism in that compound I is reconverted into native enzyme in a single two-electron reducing step [20]:

$$CPOI + Cl^- \rightarrow CPO + HOCl$$

In this contribution, we analyze the kinetics of enzyme reactions evolving according to Scheme 1, where S is a suicide substrate, assuming that j_3 inactivation constant is much higher than the k_3 catalytic constant and that the substrate concentration remains approximately constant during the assay time considered. The latter assumption can be achieved experimentally using an initial value of the enzyme concentration that is small enough compared with the initial substrate concentration. Suicide substrates may be defined in terms of their dual role as compounds that interact with enzymes by a mechanism with a branched pathway, the branches representing turnover of the substrate and inactivation of the enzyme [16,17].



Scheme 1.

The aims of the paper are to derive the time course equation for all species present in Scheme 1 under the assumption mentioned and by using these kinetic analyses to study the inactivation of the enzyme chloroperoxidase in the presence of the oxidant *tert*-butyl hydroperoxide. The chloroperoxidase is a heme enzyme exhibiting versatile properties; by its prosthetic group, an iron(III)protoporphyrin(IX), it is able to catalyze an impressive variety of oxidations using different peroxides as electron acceptors. However, the binding of the enzyme to peroxides produces an irreversible inactivation of its catalytic activities [21]. This inactivation modifies the spectral characteristics of the enzyme, so spectroscopic CPO-peroxide kinetic studies can be performed deriving the kinetic inactivation constants. The denaturation constants can be measured also with a method based on the loss of enzyme activity, using the standard assay involving the monochlorodimedone, chloride and an oxidant at pH 2.75 [22]. *Tert*-butyl hydroperoxide (*tert*-BuOOH) was chosen because with this oxidant the enzyme has low catalase activity [23].

2. Experimental

2.1. Materials

Chloroperoxidase 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 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.

2.2. Methods

2.2.1. Kinetic studies

All kinetic runs were performed in duplicate at seven different *tert*-BuOOH concentrations with three data points per concentration and were performed as follows: 5.4 μl of Fluka commercial enzyme were added to 5.4 ml of 0.1 M phosphate buffer (pH 6.00) in a quartz cell of 2 cm path length. The solution was shaken and the spectrum immediately recorded. For the kinetic study, the following standard assay mixture was used: chloroperoxidase (3 nmol), a suitable amount of *tert*-butyl hydroperoxide ($6.5 \cdot 10^{-4}$–$3.25 \cdot 10^{-2}$ M) in 0.1 M potassium phosphate buffer (pH 6.00) in a total volume of 5.4 ml. The kinetic analysis was monitored at 400 nm.

2.2.2. Stability studies

The stability of chloroperoxidase in the presence of the oxidant *tert*-BuOOH was investigated. Ten microliters of Fluka commercial enzyme were pre-incubated with different