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Biophysical Chemistry 113 (2005) 105–113

Biophysical Chemistry

www.elsevier.com/locate/bpc

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

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Received in revised form 9 August 2004; accepted 9 August 2004
Available online 27 September 2004

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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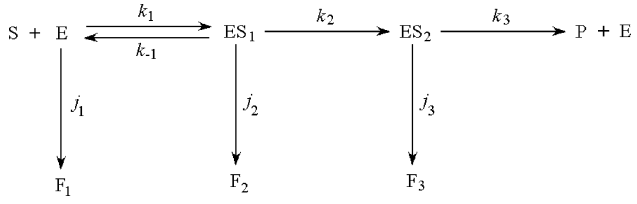
doi:10.1016/j.bpc.2004.08.003

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

concentrations of the oxidant ($1 \cdot 10^{-4}$–$7.3 \cdot 10^{-3}$ M) in 10 ml of 0.1 M phosphate buffer (pH 6.00). A control containing the same amount of enzyme and the buffer was also performed. The pre-incubations were performed in triplicate. During the pre-incubation, the tubes were shaken at 200 rev/min by using an orbital shaker. A fixed amount of these solutions 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 (0.1 mmol), monochlorodimedone (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 (ε_{278}=$1.22 \cdot 10^4$) to dichlorodimedone (ε_{278}=$1.6 \cdot 10^2$) [22].

3. Theory

Moruno-Dávila et al. [16,17] presented a kinetic analysis of a Michaelis–Menten mechanism in which the enzyme is unstable. We propose the case in which enzyme E binds to a molecule S forming a first intermediate (ES_1); then complex ES_1 is transformed in complex ES_2. The case in which the enzyme specifically binds to S (oxidant) in the absence of electron donor, and either the free enzyme E or the enzyme–substrate complexes ES_1 and ES_2 are unstable, can be described by the Scheme 1:

In Scheme 1, E is the free enzyme, S is the oxidant, ES_1 and ES_2 are the intermediates, and F_1, F_2 and F_3 are the inactive species into which the species E, ES_1 and ES_2, respectively, are transformed.

Under assumption that $k_3 \ll j_3$, the equations describing the kinetics of the species in Scheme 1 are:

$$d[E]/dt = -(k_1[S] + j_1)[E] + k_{-1}[ES_1] \quad (1)$$

$$d[ES_1]/dt = k_1[E][S] - (k_{-1} + j_2 + k_2)[ES_1] \quad (2)$$

$$d[ES_2]/dt = k_2[ES_1] - j_3[ES_2] \quad (3)$$

$$d[S]/dt = -k_1[E][S] + (k_{-1})[ES_1] \quad (4)$$

$$d[F_1]/dt = j_1[E] \quad (5)$$

$$d[F_2]/dt = j_2[ES_1] \quad (6)$$

$$d[F_3]/dt = j_3[ES_2] \quad (7)$$

$$[E]_0 = [E] + [ES_1] + [ES_2] + [F_1] + [F_2] + [F_3] \quad (8)$$

$$[S]_0 = [S] + [ES_1] + [ES_2] \quad (9)$$

The set of differential Eqs. (1)–(7) is non-linear and has, therefore, no analytical solution. However, under the assumption (10):

$$[E]_0 \ll [S]_0 \quad (10)$$

Eq. (9) simplifies to:

$$[S]_0 \cong [S] \quad (11)$$

Upon insertion of Eq. (11) into Eqs. (1)–(7), the set becomes linear. The solutions for all species are:

$$[E] = \sum \frac{\chi_E - \beta_E \delta_q}{\delta_h(\delta_h - \delta_q)} e^{\delta_h t} \qquad (h = 1,2;\ q = 1,2;\ h \neq q) \quad (12)$$

$$[ES_1] = \sum \frac{\chi_{ES_1} - \beta_{ES_1} \delta_q}{\delta_h(\delta_h - \delta_q)} e^{\delta_h t} \quad (h = 1,2;\ q = 1,2;\ h \neq q) \quad (13)$$

$$[ES_2] = \sum \frac{k_2(\chi_{ES_1} - \beta_{ES_1} \delta_q)}{\delta_h(\delta_h - \delta_q)(j_3 + \delta_h)} e^{\delta_h t} - \left(\sum \frac{k_2(\chi_{ES_1} - \beta_{ES_1} \delta_q)}{\delta_h(\delta_h - \delta_q)(j_3 + \delta_h)} \right) e^{-j_3 t}$$
$$(h = 1,2;\ q = 1,2;\ h \neq q) \quad (14)$$

$$[F_1] = \sum j_1 \frac{\chi_E - \beta_E \delta_q}{\delta_h^2(\delta_h - \delta_q)} (e^{\delta_h t} - 1)$$
$$(h = 1,2;\ q = 1,2;\ h \neq q) \quad (15)$$

$$[F_2] = \sum j_2 \frac{\chi_{ES_1} - \beta_{ES_1} \delta_q}{\delta_h^2(\delta_h - \delta_q)} (e^{\delta_h t} - 1)$$
$$(h = 1,2;\ q = 1,2;\ h \neq q) \quad (16)$$

$$[F_3] = \sum \frac{j_3 k_2(\chi_{ES_1} - \beta_{ES_1} \delta_q)}{\delta_h(\delta_h - \delta_q)(j_3 + \delta_h)} (e^{\delta_h t} - 1) + \left(\sum \frac{k_2(\chi_{ES_1} - \beta_{ES_1} \delta_q)}{\delta_h(\delta_h - \delta_q)(j_3 + \delta_h)} \right) (e^{-j_3 t} - 1)$$
$$(h = 1,2;\ q = 1,2;\ h \neq q) \quad (17)$$

where δ_1 and δ_2 are the roots of the polynomial $\delta^2 + H_1\delta + H_2$ in which:

$$H_1 = k_1[S]_0 + j_1 + j_2 + k_{-1} + k_2 \quad (18)$$

$$H_2 = k_1[S]_0(j_2 + k_2) + j_1(j_2 + k_{-1} + k_2) \quad (19)$$

Hence:

$$\delta_1 = \left[-H_1 + (H_1^2 - 4H_2)^{1/2} \right]/2 \quad (20)$$

$$\delta_2 = \left[-H_1 - (H_1^2 - 4H_2)^{1/2} \right]/2 \quad (21)$$

But for our study, we are not able to reduce these solutions (especially for F_3 species) in uni-exponential

manner, so we obtained other solutions for species $[ES_2]$ and $[F_3]$ by using cubic equation:

$$[ES_2] = \sum \frac{\chi_{ES_2} - (\gamma_q + \gamma_p)\beta_{ES_2}}{\gamma_h(\gamma_h - \gamma_q)(\gamma_h - \gamma_p)} e^{\gamma_h t}$$
$$(h = 1,2,3;\ q = 1,2,3;\ p = 1,2,3;\ h \neq q \neq p) \quad (22)$$

$$[F_3] = \sum j_3 \frac{\chi_{ES_2} - (\gamma_q + \gamma_p)\beta_{ES_2}}{\gamma_h^2(\gamma_h - \gamma_q)(\gamma_h - \gamma_p)} (e^{\gamma_h t} - 1)$$
$$(h = 1,2,3;\ q = 1,2,3;\ p = 1,2,3;\ h \neq q \neq p) \quad (23)$$

where γ_1, γ_2 and γ_3 are the roots of the polynomial $\gamma^3 + G_1\gamma^2 + G_2\gamma + G_3$ in which:

$$G_1 = H_1 + j_3 \quad (24)$$

$$G_2 = j_3 H_1 + H_2 \quad (25)$$

$$G_3 = j_3 H_2 \quad (26)$$

Hence:

$$\gamma_n = 2\sqrt{-\frac{3G_2 - G_1^2}{9}} \times \cos\left(\frac{1}{3}\arccos\left(\frac{1}{6}\frac{9G_1G_2 - 27G_3 - 2G_1^3}{\sqrt{-3G_2 - G_1^2}}\right) + \frac{2}{3}\pi n\right) - \frac{1}{3}G_1 \qquad (n = 0,1,2) \quad (27)$$

The β and χ terms for the E, ES_1 and ES_2 species are shown in Table 1.

Note that δ_1, δ_2, γ_1, γ_2 and γ_3 are all negative terms, and that the following relations exist between the roots:

$$\delta_1 + \delta_2 = -H_1 \quad (28)$$

$$\delta_1\delta_2 = H_2 \quad (29)$$

$$\gamma_1 + \gamma_2 + \gamma_3 = -G_1 \quad (30)$$

$$\gamma_1\gamma_2 + \gamma_2\gamma_3 + \gamma_1\gamma_3 = G_2 \quad (31)$$

$$\gamma_1\gamma_2\gamma_3 = -G_3 \quad (32)$$

As it appears in results the different forms of $[ES_2]$ or $[F_3]$ time dependence give the same numerical solutions by using initial conditions and kinetic parameters useful in our experimental procedures.

Table 1
Coefficients for the time kinetics of three enzyme species

α	β_α	χ_α
[E]	$-[E]_0(k_1[S]_0+j_1)$	$k_{-1}k_1[S]_0[E]_0+[E]_0(k_1[S]_0+j_1)^2$
$[ES_1]$	$k_1[S]_0[E]_0$	$-k_1[S]_0[E]_0(k_1[S]_0+j_1+j_2+k_{-1}+k_2)$
$[ES_2]$	$k_2k_1[S]_0[E]_0$	$-k_2k_1[S]_0[E]_0(k_1[S]_0+j_1+j_2+j_3+k_{-1}+k_2)$

3.1. Kinetic analysis

In many cases of practical interest, if conditions (33) and (34) are verified, $H_1 \cong G_1$ and $H_2 \cong G_2$:

$$[S]_0 \geq \frac{j_3 - j_1 - j_2 - k_{-1} - k_2}{k_1} \quad (33)$$

$$[S]_0 \geq \frac{j_1 j_3 + (j_3 - j_1)(j_2 + k_{-1} + k_2)}{k_1(j_2 + k_2 - j_3)} \quad (34)$$

So the exponential terms δ_1 and δ_2 in the kinetic Eqs. (12), (13), (15) and (22) will be express in terms of γ_1 and γ_2, respectively.

By using Lambert and Beer law for all the enzyme species, we have Eq. (35):

$$A_X = [X] l \varepsilon_X \quad (35)$$

where X is one of the enzyme species present in Scheme 1, ε_X is the coefficient of molar absorption of X specie and l is the length of the spectroscopy run.

By adding all the spectroscopic contributions deriving from Eq. (35) applied to all the enzyme species, we have the three-exponential Eq. (36):

$$A_T = C_0 + C_1 e^{\gamma_1 t} + C_2 e^{\gamma_2 t} + C_3 e^{\gamma_3 t} \quad (36)$$

where A_T is the total spectroscopic value, and C_0, C_1, C_2 and C_3 are spectroscopic linear coefficients.

By a three-exponential fitting, we can find the γ_1, γ_2 and γ_3 values, and by using Eqs. (30)–(32) we obtain G_1, G_2 and G_3 expressed by Eqs. (37)–(39).

$$G_1 = k_1[S]_0 + j_1 + j_2 + j_3 + k_{-1} + k_2 \quad (37)$$

$$G_2 = k_1[S]_0(j_3 + j_2 + k_2) + (j_1 + j_3)(j_2 + k_{-1} + k_2) + j_1 j_3 \quad (38)$$

$$G_3 = j_3 k_1(j_2 + k_2)[S]_0 + j_1 j_3(j_2 + k_{-1} + k_2) \quad (39)$$

In many cases of practical interest:

$$-\gamma_1, -\gamma_2 \gg -\gamma_3 \quad (40)$$

Eq. (40) becomes true if the relation (41) is verified [8].

$$j_1, j_2, j_3 \ll k_1[S]_0, k_{-1}, k_2 \quad (41)$$

It follows that:

$$\gamma_1 + \gamma_2 = -G_1 \quad (42)$$

$$\gamma_1\gamma_2 = G_2 \quad (43)$$

$$\gamma_3 = -G_3/G_2 \quad (44)$$

and so γ_3 is:

$$-\gamma_3 = \frac{\frac{j_3(j_2+k_2)}{(j_3+j_2+k_2)}[S]_0 + \frac{j_1 j_3(j_2+k_{-1}+k_2)}{k_1(j_3+j_2+k_2)}}{[S]_0 + \frac{(j_1+j_3)(j_2+k_{-1}+k_2)+j_1 j_3}{k_1(j_3+j_2+k_2)}} \quad (45)$$

$$V_3 = \frac{j_3(j_2+k_2)}{j_3+j_2+k_2} \quad (46)$$

$$K_3 = \frac{(j_1+j_3)(j_2+k_{-1}+k_2)+j_1 j_3}{k_1(j_3+j_2+k_2)} \quad (47)$$

At high total S concentration, Eq. (45) give a sort of Michaelis–Menten relation (48) in which γ_3 is dependent on two constants V_3 and K_3 and on the total oxidant concentration:

$$-\gamma_3 = \frac{V_3[S]_0}{[S]_0 + K_3} \quad (48)$$

by using a double reciprocal plot (49), it is easy to find all the V_3 and K_3 values.

$$\frac{1}{-\gamma_3} = \frac{K_3}{V_3}\frac{1}{[S]_0} + \frac{1}{V_3} \quad (49)$$

the V_3 and K_3 values are expressed by Eqs. (46) and (47).

3.2. Stability analyses

In a single experimental procedure, it is very hard to obtaining either j_1, j_2 or j_3 values. So, when it is possible, it would be better finding separately the various inactivation rate constants. We performed two incubation studies, with or without substrate.

For the calculus of j_1, we impose in Scheme 1 that there is no substrate electron donor; the set of analytical solutions becomes:

$$[E] = [E]_0 e^{-j_1 t} \quad (50)$$

$$[F_1] = [E]_0\left(1 - e^{-j_1 t}\right) \quad (51)$$

The enzyme activity units (EA) are proportional to the active enzyme concentration so at time zero there is a maximum in the enzyme activity units (EA_0) proportional to $[E]_0$.

$$[E] = [E]_0\frac{EA}{EA_0} \quad (52)$$

It is so possible to describe the free active enzyme concentration as a function of the total enzyme concentration, the EA at time t and the EA_0.

The j_1 rate constant can be elucidated by fitting the enzymatic activity to a general equation describing a first order decay [21]:

$$EA = EA_0 e^{-j_1 t} \quad (53)$$

Eq. (53) can be linearized as logarithmic function; j constants can be expressed as half life time $T_{1/2}$ as it appears in Eq. (55):

$$\ln(EA) = \ln(EA_0) - j_1 t \quad (54)$$

$$j = \frac{0.693}{T_{1/2}} \quad (55)$$

For the calculus of j_3, it is necessary the presence of the oxidant S; if the substrate is present, the study of the general analytical solutions is very difficult. By using Eq. (45) at high total S concentration, we obtain Eq. (56)

$$-\gamma_3 = \frac{\frac{j_3(j_2+k_2)}{j_3+j_2+k_2}[S]_0}{[S]_0 + \frac{(j_1+j_3)(j_2+k_{-1}+k_2)+j_1 j_3}{k_1(j_3+j_2+k_2)}} \quad (56)$$

So if the condition (41) is verified, Eq. (56) becomes Eq. (57)

$$\gamma_3 = -\frac{j_3[S]_0}{[S]_0 + \frac{(j_1+j_3)(k_{-1}+k_2)}{k_1 k_2}} \quad (57)$$

Using relation (41) allows one to neglect the terms involving $e^{\gamma_1 t}$, $e^{\gamma_2 t}$, $e^{\delta_1 t}$ and $e^{\delta_2 t}$ in the analytical solutions, and with Eqs. (23) and (57) we obtain:

$$[F_3] = [E]_0\left(1 - e^{-\frac{j_3[S]_0}{[S]_0 + \frac{(j_1+j_3)(k_{-1}+k_2)}{k_1 k_2}}t}\right) \quad (58)$$

Note that the $[F_1]$ and $[F_2]$ values are always negligible with respect to all other concentration values (Eq. (59));

$$[E]_0 = [F_3] + [E] + [ES_1] + [ES_2] \quad (59)$$

so the first derivative of the enzyme mass balance becomes:

$$\frac{d[F_3]}{dt} = -\frac{d[E]}{dt} - \frac{d[ES_1]}{dt} - \frac{d[ES_2]}{dt} \quad (60)$$

From Eq. (60), we note that there is a pseudo steady state between the active enzyme forms and the inactive enzyme F_3 molecule; the inactivation of the active enzyme species leads only to the formation of the inactivated form F_3.

The rate equation deriving from F_3 species is:

$$\frac{d[F_3]}{dt} = [E]_0\frac{j_3[S]_0}{[S]_0 + \frac{(j_1+j_3)(k_{-1}+k_2)}{k_1 k_2}} e^{-\frac{j_3[S]_0}{[S]_0 + \frac{(j_1+j_3)(k_{-1}+k_2)}{k_1 k_2}}t} \quad (61)$$

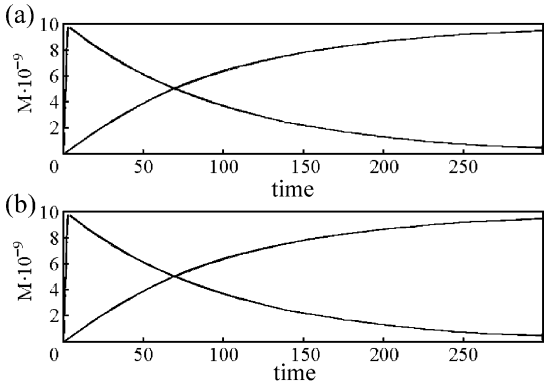


Fig. 1. Simulated time course of species $[ES_2]$ and $[F_3]$ obtained by: (a) Eqs. (14)–(17) and (b) Eqs. (22)–(23). They are used kinetic parameters value as: $[S]_0=10^{-4}$, $[E]_0=10^{-8}$, $k_1=10^5$, $k_{-1}=10^3$, $k_2=10^4$, $j_1=10^{-4}$, $j_2=10^{-4}$, $j_3=10^{-2}$.

By using Eq. (60), the Eq. (61) becomes:

$$-\left(\frac{d([E]+[ES_1]+[ES_2])}{dt}\right) = [E]_0\frac{j_3[S]_0}{[S]_0+\frac{(j_1+j_3)(k_{-1}+k_2)}{k_1k_2}}e^{-\frac{\frac{j_3[S]_0}{[S]_0+\frac{(j_1+j_3)(k_{-1}+k_2)}{k_2k_2}}}{k_2k_2}t} \quad (62)$$

The enzyme activity units (EA) are proportional to the active enzyme concentration (either free or bound); at time 0, there are a maximum in the enzyme activity units (EA_0) proportional to $[E]_0$.

$$[E]+[ES_1]+[ES_2] = [E]_0\frac{EA}{EA_0} \quad (63)$$

we express a new constant as K:

$$K = \frac{(j_1+j_3)(k_{-1}+k_2)}{k_1k_2} \quad (64)$$

By using Eqs. (62)–(64), we obtain:

$$-\frac{d}{dt}\left(\frac{EA}{EA_0}\right) = \frac{j_3[S]_0}{[S]_0+K}e^{-\frac{j_3[S]_0}{[S]_0+K}t} \quad (65)$$

where:

$$-\frac{d}{dt}\left(\frac{EA}{EA_0}\right) = v = -\frac{d}{dt}\left(\frac{[E]+[ES_1]+[ES_2]}{[E]_0}\right) = \frac{d}{dt}\left(\frac{[F_3]}{[E]_0}\right) \quad (66)$$

It is necessary to define v, expressed in terms of time^{-1}, as ratio of the reaction rate (either of formation of F_3 species or of consumption of the three species E, ES_1 and ES_2) to $[E]_0$; v is obviously related to the different substrate concentrations and time.

At zero time:

$$v_0 = \frac{j_3[S]_0}{[S]_0+K} \quad (67)$$

We define v_0 as ratio of the initial reaction rate (either of formation of F_3 species or of consumption of the three species E, ES_1 and ES_2) to $[E]_0$.

By using Eqs. (58), (59), (63) and (67), we obtain:

$$EA = EA_0e^{-v_0t} \quad (68)$$

v_0 can be obtained by linearizing Eq. (68) as logarithmic function (69):

$$\ln(EA) = \ln(EA_0) - v_0t \quad (69)$$

We can linearize Eq. (67) in any way used for the rectangular hyperbola as the Michaelis Menten one [24–28]. It was chosen the double reciprocal plot, obtaining the following Eq. (70):

$$\frac{1}{v_0} = \frac{K}{j_3}\frac{1}{[S]_0}+\frac{1}{j_3} \quad (70)$$

Eq. (70) can be used to obtaining the K and the j_3 values from the inactivation data in the presence of the substrate.

4. Results and discussion

As it appears in Fig. 1, by using kinetic parameters expressed in Fig. 1 legend, the numerical solutions either of Eqs. (14) and (22) or of Eqs. (17) and (23) are identical. This demonstrates that in the conditions similar to the our experimental ones we can use derived mathematical equations to obtain some of the kinetic constants of Scheme 1.

CPO from *C. fumago* is a heme enzyme which participates in the production of the natural product caldariomycin [29]. Although the primary biological function of CPO is chlorination [29], CPO also catalyzes

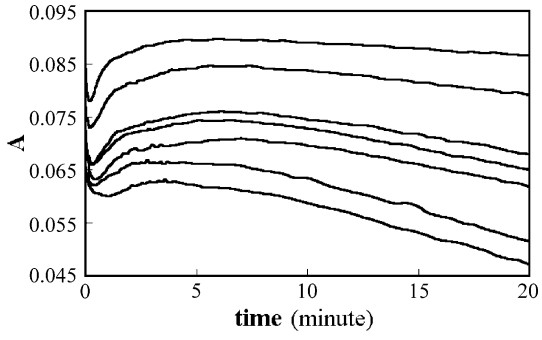


Fig. 2. Kinetic curves obtained at 400 nm in the presence of different *tert*-BuOOH concentrations.

Table 2
Kinetic parameters for enzyme species formation at various oxidant concentrations

tert-BuOOH(M)	$6.5 \cdot 10^{-4}$	$1.63 \cdot 10^{-3}$	$3.25 \cdot 10^{-3}$
γ_1 (s^{-1})	$2.51(\pm 0.43) \cdot 10^{1}$	$1.92(\pm 0.41) \cdot 10^{1}$	$1.45(\pm 0.030) \cdot 10^{1}$
γ_2 (s^{-1})	$8.00(\pm 0.43) \cdot 10^{-1}$	$4.87(\pm 0.97) \cdot 10^{-1}$	$3.64(\pm 0.25) \cdot 10^{-1}$
γ_3 (s^{-1})	$1.99(\pm 0.45) \cdot 10^{-3}$	$4.83(\pm 0.73) \cdot 10^{-3}$	$8.64(\pm 1.00) \cdot 10^{-3}$
$4.75 \cdot 10^{-3}$	$6.5 \cdot 10^{-3}$	$1.63 \cdot 10^{-2}$	$3.25 \cdot 10^{-2}$
$1.63(\pm 0.49) \cdot 10^{1}$	$1.38(\pm 016.) \cdot 10^{1}$	$1.64(\pm 0.49) \cdot 10^{1}$	$4.11(\pm 0.69)$
$3.08(\pm 0.12) \cdot 10^{-1}$	$2.74(\pm 0.31) \cdot 10^{-1}$	$2.29(\pm 0.14) \cdot 10^{-1}$	$2.09(\pm 0.11) \cdot 10^{-1}$
$1.07(\pm 0.18) \cdot 10^{-2}$	$1.28(\pm 0.09) \cdot 10^{-2}$	$1.96(\pm 0.12) \cdot 10^{-2}$	$2.20(\pm 0.04) \cdot 10^{-2}$

other oxidative reactions characteristic of other heme peroxidases, catalase and cytochrome P450 [30]. Chloroperoxidase in the presence of an oxidant forms various intermediates, the most known of which are compounds I and II. The addition of *tert*-BuOOH to native ferric chloroperoxidase leads to significant changes in the visible absorption spectrum of the enzyme. To determine the values of the inactivation constants for the enzyme, we have recorded at 400 nm the decrease of the absorbance of the native enzyme upon reaction with different concentrations of *tert*-BuOOH. The kinetic run curve seems to be a three exponential curve; so there are almost two intermediates, called in theory section ES_1 (probably compound I) and ES_2, respectively. As shown in Fig. 2 and Table 2, the formation rates of the first two intermediates are higher than the formation rate of the F_3 species.

With three-exponential fitting in the kinetic data, we can obtain the γ values at various oxidant concentrations (Table 2). We use the γ values and Eqs. (42)–(44) to obtain the G_1, G_2 and G_3 values. We see that either γ_1 or γ_2 have an experimental significance but they are not useful to obtain kinetic parameters: γ_1 values are heavily affected by diffusion so that its relation with total *tert*-BuOOH concentration is not linear as Eq. (42) shows (in condition $\gamma_1 \ll \gamma_2$), whereas the values of γ_2 are probably affected by the formation of other intermediates as shown in the Fig. 2; it should be noted that there is a hump in the ascending portions of most of the runs. The process of F_3 formation is slower than the others, indicating to be the limiting step. Further, γ_3 values are reliable, in fact, as predicted, they follow a hyperbolic kinetics in relation to *tert*-BuOOH concentrations. It is also worthwhile to note that in using the γ_3 Eq. (48) the high saturation points are more important than the lowest. By using Eq. (48), V_3 (equal to j_3) and the K_3 values were found to be $4.82(\pm 0.22) \cdot 10^{-4}$ s^{-1} and $8.02(\pm 0.34) \cdot 10^{-3}$ M, respectively (Fig. 3).

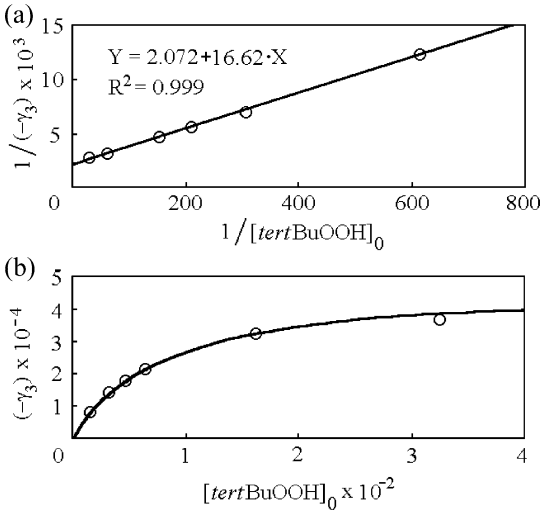


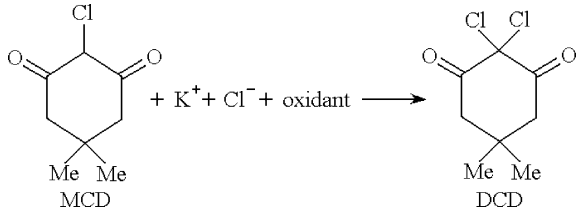
Fig. 3. Relationship between -γ_3 as and *tert*-butyl hydroperoxide: (a) double reciprocal plot and (b) rectangular hyperbola plot.

In addition, we investigated also the stability of chloroperoxidase in the presence of different concentrations of *tert*-BuOOH. The reaction rate was evaluated by using monochlorodimedone as substrate [22] (see Scheme 2).

As shown in Fig. 4, the incubation of chloroperoxidase in buffer (pH 6) leads to a first order decay of the enzyme activity in agreement with Eq. (48).

By Eq. (49), j_1 value was found to be $2.39(\pm 0.09) \cdot 10^{-7}$ s^{-1}. The $T_{1/2}$ obtained from Eq. (50) was $2.90(\pm 0.11) \cdot 10^{6}$ s. The j_1 value appears to be very small.

Furthermore, the incubation of the enzyme with different concentrations of *tert*-BuOOH results in a first order decay of its activity with respect to time, in accord with Eq. (68). The first order decay indicates that the j_3 step is the limiting step. By using Eq. (69), it is possible to obtain either v_0 or the initial enzyme activity (where EA_0 are initial enzyme activity). The v_0 is not a function of the quantity of enzyme present in the incubation mixture, provided that, $[\textit{tert}\text{-BuOOH}]_0 \gg [\text{CPO}]_0$. The v_0 and the $[\textit{tert}\text{-BuOOH}]_0$



Scheme 2.

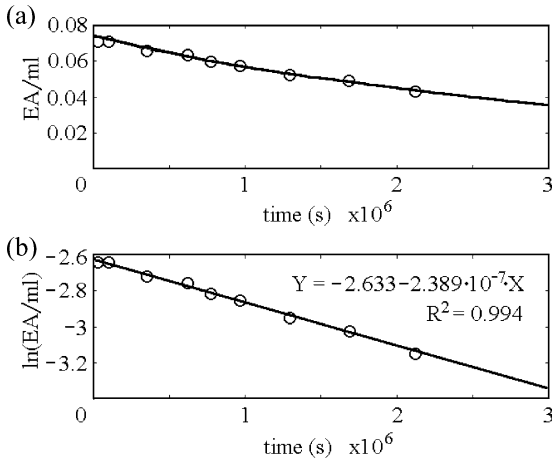


Fig. 4. (a) Enzyme activity first order decay and (b) logarithmic plot of EA/ml versus time.

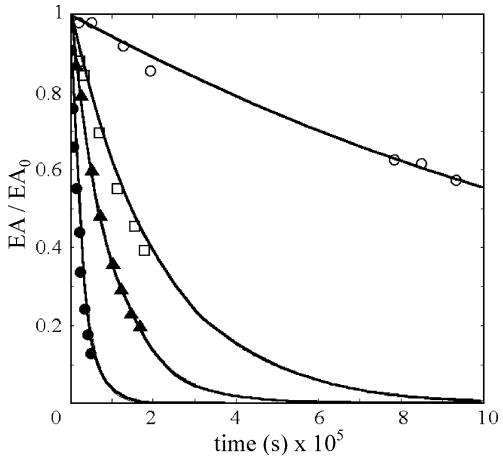


Fig. 6. Time course inactivation of CPO as a function of varying concentrations of *tert*-BuOOH. (○) 100 μM, (□) 500 μM, (▲) 1 mM and (●) 10 mM.

are linearized by double reciprocal plot (70) as shown in Fig. 5.

By Eq. (70), we determined j_3 and the K values as $4.51(\pm 0.21)\cdot 10^{-4}$ s^{-1} and $6.02(\pm 0.24)\cdot 10^{-3}$ M, respectively. It should be noted that, in both kinetic and stability studies, it was possible to use the derived equations only in condition of saturation. In fact, we obtained the kinetic constant imposing the γ_3 equation as a rectangular hyperbola. It is worthwhile to note that the values of j_3, K and V_3, K_3, obtained from kinetic and stability studies are comparable, so that one can conclude that the loss of absorbance of the enzyme in solution is due to the inactivation of the native form. We could also use Eqs. (67) and (68) and the above-mentioned values to obtain simulated inactivation curves. As shown in Fig. 6, the simulated inactivation curves perfectly overlay the experimental data. Therefore, once we have found j_3, K values (at known conditions) for the enzyme system, it is possible to estimate the half-life time. The half-life time will be important if we want to increase the product formation of a reaction in which the substrate is a suicide one.

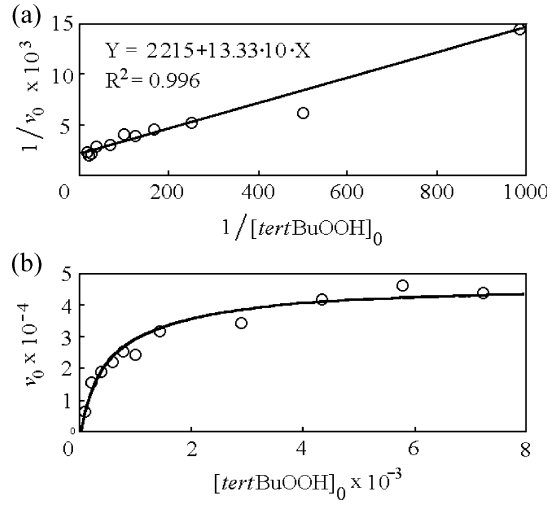


Fig. 5. Relationship between v_0 as and *tert*-butyl hydroperoxide: (a) double reciprocal plot and (b) rectangular hyperbola plot.

5. Conclusions

In this paper, a kinetic analysis and a experimental procedure for the evaluation of the kinetic inactivation parameters of Scheme 1 under assumption $k_3 \ll j_3$ have been presented.

Under conditions:

$$[E]_0 \ll [S]_0$$

$$[S]_0 \geq \frac{j_3 - j_1 - j_2 - k_{-1} - k_2}{k_1}$$

$$[S]_0 \geq \frac{j_1 j_3 + (j_3 - j_1)(j_2 + k_{-1} + k_2)}{k_1 (j_2 + k_2 - j_3)}$$

all species in the kinetic analysis present the same three-exponential terms. A kinetic analysis was proposed and V_3 and K_3 were evaluated. In cases, where uni-exponential equation describes enzyme time course inactivation, an alternative method has been presented in which j_1, j_3 and K may be evaluated. There is a good agreement between the values of constants obtained by kinetic analysis and those obtained by inactivation analysis. The inactivation analysis has the advantage that relatively lower amount of enzyme are required. In contrast, the kinetic analysis is more informative, but more that 10-fold of enzyme quantity is needed.

This mathematical approach would be useful for the cytochrome P450 inactivation study in the case that the half-life of native- and drug ligated-P450 cytochrome are very close.

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