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Suicide-peroxide inactivation of horseradish peroxidase in the presence of *Sodium n-Dodecyl Sulphate*: A study of the enzyme deactivation kinetics

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Abstract
In the presence of the anionic surfactant sodium n-dodecyl sulphate (SDS), horseradish peroxidase (HRP) undergoes a deactivation process. Suicide inactivation of horseradish peroxidase by hydrogen peroxide(3 mM) was monitored by the absorbance change in product formation in the catalytic reaction cycle. The progress curve of the catalytic reaction cycle was obtained at 27°C and phosphate buffer 2.5 mM (pH = 7.0). The corresponding kinetic parameters i.e., intact enzyme activity (α_i); the apparent rate constant of suicide inactivation by peroxide (k_i); and the apparent rate constants of enzyme deactivation by surfactant (k_d) were evaluated from the obtained kinetic equations. The experimental data are accounted for by the equations used in this investigation. Addition of SDS to the reaction mixture intensified the inactivation process. The deactivation ability of denaturant could be resolved from the observed inactivation effect of the suicide substrate by applying the proposed model. The results indicate that the deactivation and the inactivation processes are independent of each other.

Keywords: *Horseradish peroxidase, deactivation, suicide inactivation, sodium dodecyl sulphate, kinetic parameters*

Abbreviations: *HRP, Horseradish peroxidase; SDS, Sodium n-Dodecyl Sulphate; AH, Hydrogen Donor (guaiacol (orthomethoxy phenol)); E_d, Deactivated Enzyme; E_i, Inactivated Enzyme (Verdohemoprotein or Product-670); E_a, Active Enzyme;* C – I;, *Compound I;* C – II;, *Compound II; α_o, Initial Activity; α_i, Intact Activity; k_i, Inactivation Rate Constant; k_D, Deactivation Rate Constant; k_{app}, Apparent Rate Constant*

Introduction

Horseradish peroxidase (HRP, E.C. 1.11.1.7, donor H_2O_2 oxidoreductase) is a single polypeptide chain consisting of two structural domains: a rich glycosylated chain (18% by weight) that contains a single high-spin ferric protoheme (IX) prosthetic group [1–3]; and a HRP C chain (cationic isozyme) which contains 308 residues in its primary structure. The HRP C chain is the most active and the most abundant member of the peroxidase family [4–5]. HRP catalyzes the oxidation of a wide variety of aromatic compounds by hydrogen peroxide [6–7]. At high concentrations of hydrogen peroxide, peroxidase effectively converts to a verdohemoprotein referred to as P-670 as it catalyzes oxidation of a aromatic hydrogen donor (AH) in the catalytic cycle of the reaction [8–17]. According to the pyridine hemichrome spectrum, the heme of the verdohemoprotein is assumed to be identical with verdoheme. Verdoheme is known to be the main product produced from the oxidation of protoheme by hydrogen donors [18–21] and during the reaction one mole of carbon monoxide (CO) is produced for every mole of verdohemoprotein formed [22]. Verdohemoprotein is also formed once HRP is incubated at higher concentrations of both m-nitroperoxy benzoic acid and hydroperoxide (formed from indole 3-acetic acid) during the catalytic oxidation reaction [23–25].

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In the absence of reducing substrates, H_2O_2 reacts with the compound I(the product of the reaction of the first hydrogen peroxide molecule with native peroxidase) as an electron donor (reducing agent) [26–27]. Also there are some reports on the suicide inactivation of peroxidases by aromatic donors [28–32].

The reduction of compound-I is believed to precede either through a catalase-like two electron process (resulting in the formation of molecular oxygen) or by means of two single-electron transfers in which compound-II, compound-III, and the superoxide radical anion ($O_2^{\cdot -}$) are formed [5]. There are reports of extensive experimental and computational studies on the transient kinetics of formation of peroxidase intermediates through its catalytic reaction cycle [33–59]. The main feature of the transient kinetic studies is formation, identification and characterization of compounds I and II species, as well as their high potential for oxidation of some specific hydrogen donors. Also there are some reports on the transient study and identification of new kinetic intermediates [60,61] and the role of His.42 in the peroxidase catalysis and kinetics has been well considered [62–64].

It should be noted that the reaction of HRP with peroxide (>3 mM) leads to progressive inactivation of the enzyme. The investigation of both the inactivation mechanism of HRP by hydrogen peroxide (in a prolonged incubation time) [65–69] and the determination of the kinetic parameters (through the catalytic cycle reactions) have been previously reported for catalase and HRP [70–73]. The major feature of these studies allows an estimation of the inactivation rate constants and the intact activities of the enzymes by the application of direct progress curve data for from the catalytic cycle reaction.

The ionic surfactants denature globular proteins in the milimolar range [74–76] and their interactions with biomacromolecules involve both electrostatic and hydrophobic effects [77–78]. The chemical denaturation studies have indicated that ionic surfactants above the transition concentration, $[S]_{1/2}$, convert the whole population of proteins to the denatured state [79]. In most cases the denaturation process is associated with the disruption of noncovalent bonds in a tertiary structure followed by the production of a deactivated conformation [80,81]. These studies on the HRP-SDS systems indicate that SDS unfolds HRP (at $[S]_{1/2} = 0.60$ mM, 25°C and at pH = 6.4) in 2.5 mM sodium phosphate buffer. The thermodynamics of the denaturation of HRP by ionic surfactants is also reported [81–82].

Previously, we have reported various aspects of the suicide inactivation mechanism of horseradish peroxidase [73]. Our present work is an attempt to study **a**) the inactivation of HRP in the presence of SDS, **b**) the determination of the deactivation rate constants and **c**) to obtain the kinetic parameters of suicide-substrate inactivation of the enzyme by hydrogen peroxide through a steady-state mechanism.

Experimental

Materials

Horseradish Peroxidase type II (purity index of R.Z = 2.30) and sodium n-dodecyl sulphate were obtained from Sigma. Guaiacol was obtained from Fluka. The other related chemicals (analytical grades) were prepared in CO_2-free deionized water (Barnstead NANOpure D4742 E.C = 18.3 MΩ.).

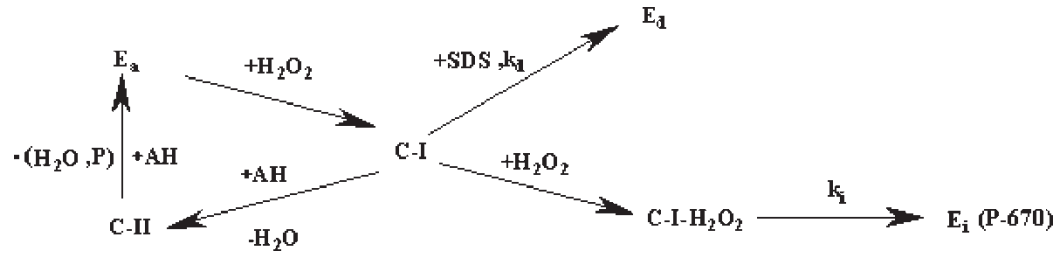
Methods

The enzyme concentrations were determined at pH 7.0 on a Shimadzu spectrophotometer model 2101 PC using an extinction coefficient of $1.02 \times 10^5\,cm^{-1}\,M^{-1}$ at 403 nm [83]. The concentration of hydrogen peroxide was estimated by measuring the absorbancy of the solution using $\varepsilon_{240} = 43.6\,cm^{-1}\,M^{-1}$ [84]. For other related calculations, a molecular weight of 42,500 was used for HRP [85]. The deactivation process at 275 nm (relating to titratable amino acid residues as chromophore), and the suicide inactivation process at 470 nm (λ_{max} for the product of the catalytic reaction) were monitored by difference spectrophotometry [86]. The details of the procedure of measuring the product concentration, the rate of reaction, and the determination of the progress curves have been described previously [73]. The progress curves were determined by the measurements of the absorbancy at an appropriate interval of 8 min apart. One minute was kept as a delay time for the addition of H_2O_2 to the HRP-AH-Surfactant system and the initiation reaction. The unreacted part of AH was obtained from the recorded absorbance data.

In order to determine the maximum elevation of absorbance(A_∞) and the value of (α_i), the reaction mixture was incubated for a prolonged time (nearly 2 h) according to the "end-point procedure" which is described elswhere [73]. In the end-point method by adding a fresh and excess quantity of HRP solution to the incubated mixture, values of A_∞, the unreacted parts of AH and also the intact activity of the enzyme (α_i), could be estimated. Intact activity (α_i) is defined as the enzyme activity before it is exposed or reacted with the substrate.

Results and discussion

It is generally accepted that ionic surfactants denature globular proteins by means of electrostatic and hydrophobic forces through a reversible denaturation process [76]. At a concentrations higher than $[S]_{1/2}$ the process is considered as in a dominant one way direction. Under such conditions, the enzyme loses its



Scheme 1. Inactivation/deactivation system under study.

activity because of the denaturing effect of the surfactants. Hence, the course of the reaction would be considered as a deactivating process because of the formation of denatured and deactivated forms of the enzyme (E_d in Scheme 1).

On the other hand, it is deemed that HRP may go through an additional kinetic mechanism of inactivation namely "suicide-substrate inactivation" besides its two described major catalytic pathways [5,69,73,87,88]. So, the principal and central role of the compound I-peroxide complex is well illustrated in suicide substrate inactivation of the enzyme. The product of the inactivation process (**P-670**) is a catalytically inactive form of HRP (E_i in Scheme 1) [65,73,89,90].

Deactivation of HRP by SDS

The investigation of the deactivating effect of SDS on HRP was carried out according to the Maehly method [83]. The activity measurements were conducted in the presence of various concentrations of the surfactant, using guaiacol as the reducing substrate. Figure 1, shows the activity dependence of HRP versus the surfactant concentration and indicates the reduction of the enzyme activity (nearly 80%) in the presence of a low concentration of SDS (0.60 mM). The previous studies of chemical denaturation of HRP by SDS give a transition concentration of $[SDS]_{1/2} = 0.60$ mM [81]. The binding studies of interaction of SDS with HRP show that at the transition concentration of $[SDS]_{1/2}$, 21 moles of SDS are bounded to one mole of HRP [81]. Since low concentrations of HRP(10^{-6} mM) have been used in the catalytic reaction mixture, SDS concentration has been maintained constant during the deactivation reaction.

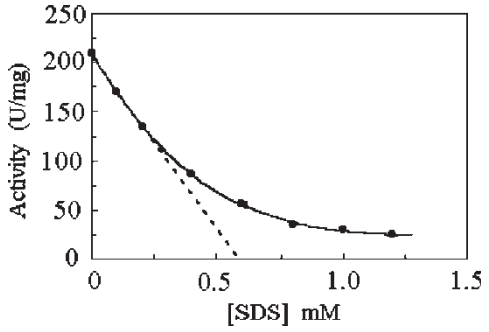


Figure 1. Activity dependence of HRP on SDS concentration. Activity was assayed using the guaiacol test [86]. SDS concentration was far below the critical micelle concentration (CMC) in all experiments. [HRP] = 1 nM, [peroxide] = 0.17 mM, [guaiacol] = 16.7 mM, lag time = 7 Sec., pH = 7.0 of phosphate buffer 2.5 mM.

Figure 2, indicates a typical progress curve for the deactivation of HRP by SDS. The experimental data could be fitted into a polynomial of the general form of [91–92]:

$$c_t = \sum_{i=1,2,\ldots} a_i e(-t/\tau_i) \qquad (1)$$

Once, the measured physical parameter is the absorbancy, the equation rearranges to:

$$A_t = a_1 e^{(-k_1 t)} + a_2 e^{(-k_2 t)} \qquad (2)$$

Where c_t is the concentration at time "t", a_1 and a_2 are the amplitudes of the function, τ_i's are the relaxation times corresponding to the forward rate constants of

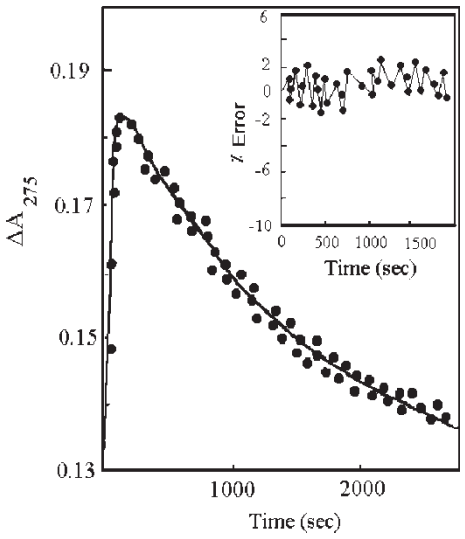


Figure 2. Progress curve for deactivation of HRP by SDS. Lag time = 10 s. Experimental conditions as noted in legend for Table I.

Table I. Kinetic parameters of deactivation of HRP (5.0×10^{-3} mM) by sodium dodecyl sulphate (0.60 mM) at 27°C, pH = 7.0, phosphate buffer (2.5 mM). Parameters are obtained by fitting of the experimental data of Figure 1 into Equation (2) with an average minimum error of 10^{-4}.

a_1	a_2	τ_1 (min)	τ_2 (min^{-1})	k_1 (min^{-1})	k_2 (min^{-1})
6.05×10^{-2}	-9.10×10^{-3}	1.62	166.40	$6.17 \times 10^{-1} \pm 2.0 \times 10^{-3}$	$6.10 \times 10^{-3} \pm 4.0 \times 10^{-4}$

τ_1 and τ_2 are obtained mathematically from fitting experimental data (Figure 2) using Equation (2).

the following strong deactivation mechanism:

$$\text{Native (N)} \quad \underset{\rightarrow}{k_1} \quad \text{Intermediate (I)} \quad \underset{\rightarrow}{k_2} \quad \text{Denatured (D)} \tag{a}$$

Here k_1 and k_2 are the forward rate constants for the strong or fast denaturation pathway.

A computer program was prepared and designed (applying the **MATLAB** software) to fit the experimental data in equation (2). The program runs in a manner such that the experimental data coincides on the calculated curve. Also the program displays the corresponding polynomial function, properly. By applying the nonlinear least squares method, the function is minimized with respect to four parameters (a_1, a_2, τ_1, τ_2) resulting in a minimum error of fitting. Figure 2, indicates a two-phase kinetic mechanism associated with a kinetically stable intermediate (I) in which the fitting of the experimental data is well satisfied for a two-terms exponential equation. Figure 2 (inset), also indicates the relative errors attributed to the experimental and the theoretical progress curves. At the presence of $[SDS] \geq [SDS]_{1/2}$, a strong denaturation occurs. The rate constants (k_1, k_2) as previously described are forward rate constants of the deactivation or denaturation process and their values could be estimated from the corresponding relaxation times as [91]:

$$\text{Native (N)} \quad \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \quad \text{Intermediate (I)} \quad \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \quad \text{Denatured (D)} \tag{b}$$

$$\tau_{\text{fast}}^{-1} = k_2 + k_{-2}; \tag{3}$$

$$\tau_{\text{slow}}^{-1} = k_{-1} + k_{-1}[k_2/(k_2 + k_{-2})]$$

Also k_{-1} and k_{-2} are the backward rate constants of the renaturation pathway. Backward rate constants may be estimated by monitoring the renaturation process at low concentrations of the surfactants (or by dilution of the HRP/SDS solution). In this study in order to combine and simultaneously investigate the deactivation and inactivation processes, only the conditions for the strong denaturation pathway are used and the backward pathway is not considered.

According to the steady state approximation, refolding of the intermediate conformation to the native state is assumed to be the rate-determining step. On the other hand, under strong denaturation conditions (high surfactant concentration) we may have: $k_2 >> k_{-2}$, $k_1 >> k_{-1}$ and $\tau_{\text{fast}}^{-1} = k_2$ and $\tau_{\text{slow}}^{-1} = k_1$

The parameters of the deactivation process are tabulated in Table I.

Suicide-peroxide inactivation of HRP in the presence of SDS

Based on our previous report [73], an addition of a new concurrent reaction (deactivation reaction of HRP by SDS) in a "suicide-substrate inactivation system is summarized in the following scheme.

According to Scheme 1, three major reactions could be attributed to the reaction system:

$$E_a \xrightarrow{(H_2O_2)k_i} E_i \tag{c}$$

$$E_a \xrightarrow{(SDS)k_d} E_d \tag{d}$$

$$AH \xrightarrow{(H_2O_2 \cdot E_a)\alpha} P \tag{e}$$

Reactions **c** (Inactivation process) and **d** (deactivation process) are of concurrent types, and the reaction **e** (oxidation of guaiacol by peroxide) is the catalyzed reaction used to monitor the deactivation or inactivation processes.

E_a, E_d, E_i, AH, and P denote the forms of active enzyme, deactivated enzyme, inactivated enzyme, hydrogen donor (reductant-substrate), and the product (tetraguaiacol) respectively. The k_i, k_d, and **α** denote the rate constant of inactivation, the rate constant of deactivation by surfactant, and the rate constant of oxidation of the hydrogen donor, respectively. In the catalytic reaction cycle, the active enzyme species are free active enzyme (E_a), C-I and C-II. The rate constants of formation of C-I, C-II and E_a are extremely large compared with the k_d and k_i values. Accordingly, the active whole enzymes are in the forms of C-I undergoing the inactivation or deactivation process based on the operating conditions. The following conditions are recommended for the simultaneous progress of the reactions:

(a) Higher concentration of hydrogen peroxide with respect to the concentration of hydrogen donor (AH)

(b) A restricted concentration of hydrogen donor ("benign substrate", a substrate which does not damage the enzyme); so the enzyme could be kept far from its saturation by the donor, and
(c) Sufficient high concentration of surfactant (higher than the transition concentration, $[S]_{1/2}$).

The reaction is essentially first order in relation to the hydrogen donor and a linear dependence on the initial velocity of the AH concentration accordingly to our previous report [73]. As a result, the rate of reaction **c** seems to be proportional to the active enzyme concentration; and the reaction **d** indicates the deactivation branch (a parallel reaction to the inactivation reaction **c**). The concept of the concurrent (parallel) reactions associated with a steady-state treatment was used to derive the integrated kinetic equation. In principle, by monitoring AH concentration in the catalytic reaction cycle, one can estimates the extent of decreasing levels of active forms of the enzyme (due to the suicide inactivation and surfactant deactivation processes). The current model provides the advantages of monitoring the inactivation and deactivation processes of enzymes without requiring the measurement of the concentrations of enzyme species (E_a, C-I, C-II, E_i or E_d). In fact, the overall concentration of active enzyme species, $\boldsymbol{\alpha}$, (in the form of C-I) defines the first order rate law at any time ($\boldsymbol{\alpha} = \boldsymbol{\alpha}_o \mathbf{e}^{(-k_i \cdot t)}$). Thus, it is only sufficient to estimate the initial active enzyme concentration, $\boldsymbol{\alpha}_o$, which is being specified under the experimental conditions. $\boldsymbol{\alpha}_o$ can be obtained by fitting the experimental data into the integrated kinetic equation for the process. The rate of the consumption of reducing substrate (AH) and the overall rate of conversion of active enzyme (E_a) to both the inactivated (E_i) and deactivated (E_d) forms enables one to obtain the differential kinetic equation as well as the modified integrated kinetic relationship (according to our previous model) [70–73]:

$$[AH]_t = [AH]_o \mathrm{Exp}(\alpha_o / k_{app})(e^{-k}app^{\cdot t} - 1) \quad (4)$$

$$k_{app} = (k_i + k_d) \quad (5)$$

Where α_o is the value of α at time (t) = 0. $[AH]_t$ and $[AH]_0$ are the molar concentrations of AH at time t and t = 0, respectively. Also k_{app} is the overall apparent rate constant for the two simultaneous and concurrent deactivation and inactivation processes (reactions **c** and **d**). Equation (4) can be used for the determination of $\boldsymbol{\alpha}_0$ and k_{app} in a non-linear regression manner by fitting the experimental data to the equation. Common computer software such as **EUREKA** can be used for this purpose. In the absence of surfactant, $k_d = 0$, Equation (4) is simplified, accordingly to our previous report [73]. Hence, the inactivation rate constants, k_i, in Equation (5) could be determined for suicide inactivation of HRP either in the absence or in the presence of SDS.

On the other hand, at low concentrations of H_2O_2 (120 μM), the normal catalytic cycle proceeds without the suicidal inactivation effect ($k_i = 0$), and Equation (6) would characterize the individual deactivation behavior of the surfactant as:

$$[AH]_t = [AH]_o \mathrm{Exp}(\boldsymbol{\alpha}_o / k_d)(e^{-k}d^{\cdot t} - 1) \quad (6)$$

Under such conditions, the level of the active enzyme decreases through reaction **Sd**.

Figure 3, shows the initial rate of the reaction (in the first order region of the Michaelis-Menten pattern) as a function of surfactant concentration where a linear dependency is observed (up to $[SDS]_{1/2}$). Figure 3 (inset), also shows a linear relation between the initial velocity of the catalytic reaction and the concentration of AH (up to 7 mM) at the presence of SDS. This indicates that the order of the reaction in relation to AH is first-order at the concentration of AH (about 100 μM). Figure 4, shows the various types of the progress curves at different concentrations of hydrogen peroxide. Figure 4(inset), also shows the progress curves of the reaction system in the presence of various concentrations of SDS. The details of the experiments are given in the legend to the figures.

Upon solving Equation (1), simultaneous estimation of k_i and k_d produces identical results. This fact indicates that reactions **c** and **d** in Scheme 1 could be considered independent of one another. Figure 5, shows the linear dependence of k_d on the surfactant

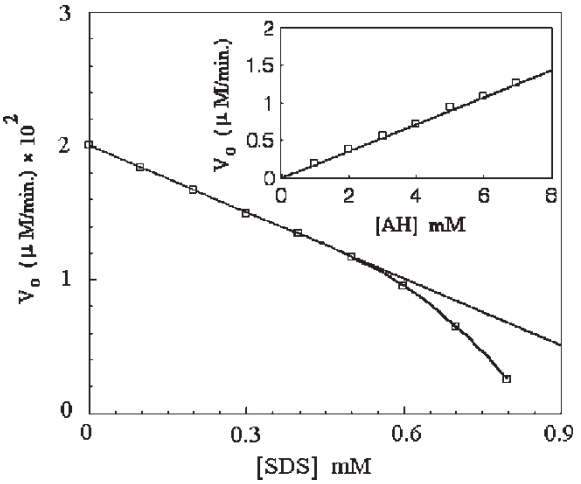


Figure 3. Initial rate of the catalytic reaction as a function of SDS concentration. The rate of reaction was measured in the presence of various concentrations of SDS for a reaction time of 30 s following a 5 s delay time. The reactions were started by addition of 10 μl aliquots of 3.0 mM H_2O_2 to 990 μl of 0.10 mM guaiacol solution in the presence of HRP having an initial activity of 3.0×10^{-3} min^{-1}. at pH = 7.0 and 27°C. **Inset**: Initial rate of reaction as a function of AH concentration in the upward region (1–7 mM of AH) in the presence of 0.60 mM SDS. The other experimental conditions are as above.

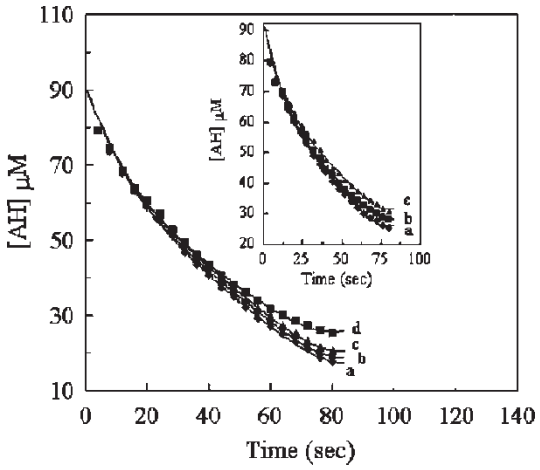


Figure 4. Typical progress curves obtained by monitoring of AH concentration. The reactions were started between AH (100 μM), about 13 nM HRP having an initial activity of 0.118 min^{-1}, and **a**) 6.0 mM H_2O_2; $k_i = 0.110\,min^{-1}$, **b**) 4.0 mM H_2O_2; $k_i = 0.072\,min^{-1}$ and **c**) 3.0 mM H_2O_2; $k_i = 0.040\,min^{-1}$. The AH concentration was monitored indirectly using the absorbency of the reaction mixture at 470 nm during about 8 min. k_i values were obtained by non-linear fitting of the experimental data into the Equation (3). **Inset**: Typical progress curves in the presence of various concentrations of SDS. Deactivation rate constants were obtained by fitting the progress curves data into Equation (4). [HRP] ≈ 13 nM, [guaiacol] = 100 μM. **Deactivation Experiments**: **a**) normal progress curve without suicide and deactivating effects, [peroxide] = 0.12 mM **b**) [peroxide] = 0.12 mM; [SDS] = 0.30 mM **c**) [peroxide] = 0.12 mM; [SDS] = 0.60 mM. **Inactivation Experiment**: [peroxide] = 3.0 mM; [SDS] = 0.60 mM. In order to reach the stationary state of the reaction, a delay time of 60 s was used for recording and processing the progress curves data.

concentration. The transition concentration of surfactant ($[SDS]_{1/2}$) is selected as the suitable concentration for the deactivation kinetic studies [79]. This transition concentration coincides with the corresponding x-intercept point in Figure 1 [79–80].

The precision of Equation (4) for the expression of behavior of the reaction system may be evaluated in different ways based on the predictions of linear forms of the equation (results not shown). Details of the procedures have been explained previously [73].

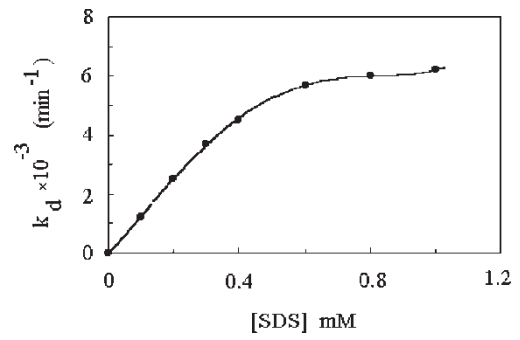


Figure 5. Variation of k_d with SDS concentration. k_d values were obtained from Equations (3) and (4) and the experimental conditions are the same as shown in the legend for Figure 4.

Therefore the following results can be extracted:

(a) The calculated k_d and k_i values are independent of the selected segments of the progress curve.
(b) k_d and k_i values remain constant once the initial values of either enzyme activity or the concentration of AH changes in an acceptable range.
(c) At an infinite time, the relation between the remaining concentration of substrate, $[AH]_\infty$, and the other variables can be predicted from equation (4).

The reliability and the accuracy of such predictions were examined for the deactivation mechanism. The results were compatible. These findings show clearly that:

(1) The change of the initial (intact) activity of peroxidase should lead to expectation of changes in [AH], and
(2) Keeping the conditions of $[H_2O_2]/[AH] = 30$, [SDS] >> [HRP], the optimum conditions for monitoring the simultaneous suicide inactivation, and the surfactant denaturation of HRP are provided The effect of variation of H_2O_2 concentration on k_i and k_{app} illustrates a linear proportionality of the rate of inactivation to the concentration of suicide substrate, H_2O_2 (data not shown) [73]. On the other hand, compound-I undergoes a deactivation effect induced by the denaturing power of the surfactant. As was described earlier, binding studies of the interaction between HRP and SDS have shown that at the transition concentration where $[SDS]_{1/2} =$ 0.60 mM, 21 moles of SDS bind to one mole of HRP. Therefore, at the designated conditions of a reaction where $[HRP] = 4.2 \times 10^{-4}$ mM and [SDS] = 0.60 mM, the bound surfactant (21 moles) can be neglected or ignored in relation to the total moles of surfactant per moles of HRP (~1430).

Under certain concentrations of H_2O_2 and the surfactant, the rate of inactivation and deactivation reactions (two independent and first order concurrent reactions) may be considered as a measure of the compound-I level in the reacting system. Thus, k_i and k_d values could be affected by factors that alter the level of compound-I or its availability in the catalytic cycle as a free form. The parameters of interest were found to be: $k_i = 3.990 \times 10^{-2} \pm 0.032 \times 10^{-2}\,min^{-1}$, $k_d = 5.700 \times 10^{-3} \pm 0.041 \times 10^{-3}\,min^{-1}$, and $\alpha_i = 0.118 \pm 0.0093\,min^{-1}$ at 3 mM H_2O_2, 27°C and sodium phosphate buffer 2.5 mM, pH = 7.0 for the SDS/HRP/H_2O_2/guaiacol system.

Recently Tams and Welinder [93] have reported the first order kinetics of unfolding HRP by guanidine

hydrochloride (Gdn-HCl). Their results show that in a two-state mechanism with a concentration of [GdnHCl] = 5.2 M, the native state converts to an unfolded one (native state converts to an unfolded one ($N \xrightarrow{k_u} U$)). Thus the unfolding rate constant (k_U) is equals to $5 \times 10^{-4}\,s^{-1}$. However, close values of k_2 and k_d indicate that the rate determining step of the kinetic mechanism of HRP deactivation by SDS based on the relaxation time method is the $I \xrightarrow{k_2} D$ step (see reactions **a** and **b**). Thus regarding the steady-state approximation method, the k_2 value corresponds to the observed kinetic rate constant of deactivation of HRP by SDS (k_d).

In order to avoid any problems such as: **a**) long incubation time, **b**) requirement for accurate determination of activity which is practical and devoid of time and cost consuming experimental procedures, **c**) the need for a long experimental time for performing the deactivation experiments (*e.g.* about several hours), and **d**) unavailability of a suitable and accurate equation for fitting the experimental data and extracting the appropriate kinetic parameters, here, our simple model presents an accurate and low cost method with a short experimental time. The model can give precise kinetic parameters for simultaneous determination of suicide-substrate inactivation and surfactant deactivation of an enzyme, using mathematically proved integral kinetic equations.

Concluding remarks

Our new modified model for a bi-substrate enzyme together with its newly derived kinetic relationships indicates a promising means for characterizing the kinetic behavior of HRP through a deactivation, inactivation or a mixed simultaneous deactivation/inactivation mechanism. Our method enables determination of the rate constants of the reactions (conversion of the active HRP to the deactivated and inactivated forms) by performing simple experiments.

The determination of k_d in a reacting system is useful for studying the effect of surfactants on the dynamics of enzymes. Therefore, it is possible to apply and extend this model for other multi-substrate enzymes (involving at least one benign substrate). Furthermore suicide and deactivation kinetic parameters can be estimated simultaneously.

In general, this mechanism is promising if:

(1) The enzyme is essentially far from saturation by its benign substrate (AH)
(2) The enzyme decays in a first order manner, and
(3) The order of the reaction in relation to the surfactant or suicide substrate is zero order.

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