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Steady-state kinetics of *Roystonea regia* palm tree peroxidase

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ABSTRACT

Royal palm tree peroxidase (RPTP) has been isolated to homogeneity from leaves of *Roystonea regia* palm trees. The enzyme purification steps included homogenization, $(NH_4)SO_4$ precipitation, extraction of palm leaf colored compounds and consecutive chromatography on Phenyl-Sepharose, TSK-Gel DEAE-5PW and Superdex-200. The novel peroxidase was characterized as having a molecular weight of 48.2 ± 3.0 kDa and an isoelectric point pI 5.4 ± 0.1. The enzyme forms dimers in solution with approximate molecular weight of 92 ± 2 kDa. Here we investigated the steady-state kinetic mechanism of the H_2O_2-supported oxidation of different organic substrates by RPTP. The results of the analysis of the initial rates vs. H_2O_2 and reducing substrate concentrations were seen to be consistent with a substrate-inhibited Ping-Pong Bi-Bi reaction mechanism. The phenomenological approach used expresses the peroxidase Ping-Pong mechanism in the form of the Michaelis-Menten equation and affords an interpretation of the effects in terms of the kinetic parameters $K_m^{H_2O_2}$, $K_m^{AH_2}$, k_{cat}, $K_{SI}^{H_2O_2}$, $K_{SI}^{AH_2}$ and of the microscopic rate constants k_1 and k_3 of the shared three-step peroxidase catalytic cycle. Furthermore, the concentration and time-dependences and the mechanism of the suicide inactivation of RPTP by hydrogen peroxide were studied kinetically with guaiacol as co-substrate. The turnover number (r) of H_2O_2 required to complete the inactivation of the enzyme was 2154 ± 100 and the apparent rate constants of catalysis 185 s^{-1} and 18 s^{-1}.

Keywords: *Roystonea regia*; Peroxidase; Steady-State Kinetics; Substrate Inhibition; Mechanism-Based Inactivation Kinetics; Hydrogen Peroxide

1. INTRODUCTION

Peroxidases (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are widely distributed in the living world and are involved in many physiological processes. The oxidation of many biological substances in body fluids leads to the production of a certain amount of hydrogen peroxide. Thus, although the function of peroxidases is often seen mainly in terms of causing the conversion of toxic H_2O_2 to H_2O, their wider participation in other reactions, such as cell wall formation, lignification, the protection of tissues from pathogenic microorganisms, suberization, auxin catabolism, defense, stress, etc., should not be overlooked [1].

Apart from their biological functions, peroxidases are important as regards many biotechnological applications. This group of enzymes, especially those of plant origin, enjoys widespread use as catalysts for phenolic resin synthesis [2,3] as indicators for food processing and diagnostic reagents [4,5], and as additives in bioremediation [6,7]. Under certain specific conditions, the radicals formed can break bonds in polymeric materials, destroying them [8].

Peroxidases reduce hydrogen peroxide and oxidize a large number of compounds, including phenols, aromatic amines, thiosanisoles, halide and thyocianate ions, and fatty acids. Their selectivity with respect to reducing substrates depends on their specific type [9]. Since they are able to oxidize a broad variety of organic and inorganic substrates [8], it is somewhat difficult to determine which substrates are physiologically relevant for plant peroxidases.

Peroxidases have been identified throughout the plant kingdom, but that from horseradish root (HRP), and in particular the slightly basic C isoenzyme (HRPC), is the one that has attracted the most attention. Despite this, recently more data have become available regarding

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peroxidases from other plants, such as peanut [10], barley [11], tea [12], *Arabidopsis thaliana* [13], and palm trees [14-18].

The shared three-step catalytic cycle of peroxidases, involving different intermediate enzyme forms, is known as the Poulos-Kraut mechanism [19,20]. Catalysis is initiated by the binding of H_2O_2 to the high-spin ferric haem iron of resting peroxidase, followed by heterolytic cleavage of the peroxide oxygen-oxygen bond under the influence of highly conserved histidine and arginine residues at the active site [21]. The haem undergoes a two-electron oxidation, forming an intermediate (Compound I) that contains an oxyferryl species (Fe(IV)=O) and a porphyrin π-cation radical. As the co-product of the reaction, a water molecule is generated. Completion of the catalytic cycle usually involves two successive single-electron transfers from separate reducing substrate molecules to the enzyme. The first reduction, of the porphyrin π-cation radical in Compound I, yields a second enzyme intermediate, Compound II, which retains the iron in the oxyferryl state [22]. Under steady-state conditions, the reduction of Compound II, to recover the ferric enzyme, is often rate-limiting. The extremely reactive free radicals released from the catalytic cycle often condense spontaneously, giving rise to polymers.

The peroxidase cycle is generally considered irreversible. Nevertheless, it is unquestionable that adsorption complexes between the enzyme and its substrates exist physically [23]. The microscopic constants governing the equilibrium between aromatic compounds and peroxidase have been estimated. Even though the co-substrates (donor or H_2O_2) in the enzyme modulate each other's affinity, it is possible for the mechanism to proceed via random binding. This observation, together with retain special kinetic features [24], supports the notion that there is no need for the peroxide to bind to the enzyme prior to donor adsorption.

Here we investigated the kinetic mechanism of the H_2O_2-supported oxidation of different organic substrates by means of a novel plant peroxidase from the *Roystonea regia* palm tree (RPTP).

Among the broad variety of organic and inorganic substrates of peroxidases, in the present work we explored six organic chromogenic substrates: three phenolics, guaiacol (2-metoxiphenol), catechol (2-hydroxyphenol) and ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid), and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), often used as a reference substrate, o-dianisidine and o-phenylendiamine, the latter three suitable for use in ELISA procedures that employ peroxidase conjugates [25-28].

Since these oxidation reactions exhibit Michaelis-Menten saturation kinetics with respect to both substrates, the chromogenic substrate and H_2O_2, the system was amenable to steady-state kinetic experiments, which were used to deduce the kinetic mechanism of the reaction following methodologies established for two-substrate enzyme systems [29]. The results of the initial-rate and inhibition studies carried out here indicate that the H_2O_2-supported oxidation of different organic substrates catalyzed by this peroxidase proceeds via a Ping-Pong Bi-Bi mechanism mediated by the oxidized enzyme intermediate Compounds I and II.

Palm peroxidase catalyzes the oxidation reactions of a large variety of reducing substrates, using H_2O_2 as oxidizing agent [19,30,31]. In the absence of reducing substrates, excess H_2O_2 leads to the inactivation of the enzyme, in this case acting as a suicide substrate of peroxidase and being irreversibly bound to its active site [32, 33]. Despite this, it has been suggested that HRP inactivation by hydrogen peroxide would be due to the formation of one or several non-active enzyme products, probably through the formation of Compound III (peroxyl-FeIII porphyrin) [34,35].

The oxidative inactivation of peroxidases is mechanism-based. The molecular mechanism driving this hydrogen peroxide-mediated inactivation is extraordinarily complex because an array of reactions can occur subsequent to the reaction of the haem iron with the hydroperoxide. Regardless of the differences among the peroxidases, a common inactivation mechanism involving several stages can be proposed. In the absence of substrate, or when they are exposed to high hydrogen peroxide concentrations, peroxidases show the kinetic behavior of suicide inactivation, in which hydrogen peroxide is the suicide substrate that converts Compound II into a highly reactive peroxy-iron(III) porphyrin free-radical termed Compound III [36]. Compound III does not form part of the peroxidase cycle, but is produced under excessive exposure of protonated Compound II to oxidative species in a reaction that is partially mediated by free superoxide radicals [37].

Despite representing different structural groups, kinetic models for the hydrogen peroxide-mediated inactivation of horseradish peroxidase (HRP) [33], ascorbate peroxidase (APX) [38], peroxidase from the Royal Palm Tree (RPTP) [16], microperoxidase-11 [39] and *Chamaerops excelsa* peroxidase (CEP) [40] are similar in the sense that they are time-dependent and exhibit saturation kinetics. From the inactivation stoichiometry, it has been concluded that for APX only 2.5 molecules of hydrogen peroxide are required per active site for the inactivation form to be generated [38], in contrast to the 265 molecules required for HRP [33]. This difference is due to the low catalytic activity of HRP, which is absent in APX [41]. For APX peroxidase, inactivation is correlated with enzyme bleaching, suggesting haem destruction [38]. Another factor in this difference is the glycosylation of

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the enzyme, which seems to be important in protecting the enzyme from inactivation [33].

2. EXPERIMENTAL

2.1. Materials

Analytical or extra-pure grade polyethyleneglycol (PEG), guaiacol, catechol, ferulic acid, ABTS, o-dianisidine, o-phenylendiamine, ammonium sulfate, sodium phosphate and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis MO, USA) and were used without further purification. H_2O_2 was from Merck (Darmstadt, Germany). Superdex-200 columns and Phenyl-Sepharose CL-4B columns were from GE Helthcare Bio-Sciences AB (Uppsala, Sweden). TSK-Gel DEAE-5PW was purchased from Tosoh Co. (Tokyo, Japan). Cellulose membrane tubing for dialysis (avg. flat width 3.0 in) was purchased from Sigma Chemical Co.; slide A-lyzer dialysis cassettes (extra-strength, 3 - 12 mL capacity, 10.000 MWCO) were form Pierce Biotechnology, Inc. (Rockford, IL, USA) and filter devices (Amicon Ultra Cellulose 10.000 MWCO, 15 mL capacity) were from Millipore Corp. (Billerica, MA, USA). All other reagents were of the highest purity available. The water used for preparing the solutions was double-distilled and then subject to a deionisation process.

2.2. Enzyme Purification

RPTP was purified from palm tree *Roystonea regia* as described [15,17] but with some modifications. The purity of the RPTP was determined by SDS-PAGE as described by Fairbanks *et al*. [42] on a Bio-Rad Minigel device using a flat block with 12% polyacrylamide concentration; by gel filtration, which was performed using a Superdex 200 10/30 HR column in an FPLC Amersham Äkta System; and by UV-visible spectrophotometry (RZ = A_{403}/A_{280} = 2.8 - 3.0). Analytical isoelectrofocusing was performed on a Mini IEF cell model 111 (Bio-Rad Laboratories, Hercules, CA, USA) using Ampholine PAG-plates, pH 3.5 - 9.5 (GE Healthcare Biosxiencies AB, Upsala, Sweden). The electrophoretic conditions and Silver Staining Kit Protein were as recommended by manufacturer. The standards used were from a broad-range pI calibration kit (4.45 - 9.6) from Bio-Rad Laboratories (Hercules, CA, USA.).

2.3. Enzymatic Activity of RPTP

The initial rates of appearance of the products of oxidation of different substrates (guaiacol, catechol, ferulic acid, ABTS, o-dianisidine, o-phenylendiamine) due to the catalytic action of RPTP in the presence of H_2O_2 were measured by electronic absorption spectroscopy at the characteristic wavelengths of such products (470, 295, 318, 414, 420 and 445 nm, respectively) [30]. The reactions, initiated by the addition of RPTP, were performed at 25˚C in 20.0 - 30.0 mM universal buffer, containing variable concentrations of the reducing substrate at fixed H_2O_2 concentration and *viceversa*. The reactions were carried out at the optimal pH for each substrate, 6.9 for guaiacol, 3.5 for catechol, 4.0 for ferulic acid, 3.0 for ABTS and 6.0 for o-dianisidine and o-phenylendiamine [43].

The concentration of peroxidase was measured spectrophotometrically at 403 nm, using the experimentally determined extinction coefficient value of 60.8 ± 2.3 $mM^{-1}\cdot cm^{-1}$ for the protein monomer [43].

To determine the microscopic rate constants and other kinetic parameters for the oxidation of the substrates by RPTP in the presence of H_2O_2, the mathematical treatment of Morales and Ros-Barceló [44] was applied. The initial reaction rates were obtained from the kinetic runs and fitted *vs.* substrate concentration, at fixed H_2O_2 concentration, and *viceversa*, according to the generally accepted two-substrate Ping-Pong mechanism for the peroxidases [19,20].

2.4. Inactivation Experiments

RPTP was inactivated at 25˚C in 10 mL incubations of universal buffer, pH 6.5, containing a fixed amount of the enzyme (136 nM). The reactions were started by the addition of H_2O_2 (over a range of concentrations). At specified time intervals, 5 - 20 µL aliquots of the incubation mixtures were transferred to cuvettes containing 2 mL of an assay mixture composed of 18 mM guaiacol and 4.9 mM H_2O_2. Peroxidase activity was measured by the increase in absorbance at 470 nm [30]; neither the substrate nor the RPTP present in the assay mixture interfered with the measurement. A minimum of three incubation assays for each peroxide concentration were performed. The residual enzymatic activity (A_R) was taken as the enzymatic activity remaining (A_t) as a percentage of the initial activity (A_o). The residual peroxidase activity was assayed at different times and after 24 h of incubation.

3. RESULTS AND DISCUSSION

3.1. Enzyme Purification

RPTP was purified to homegeneity with a high yield from palm tree *Roystonea regia* leaves. The purification steps and their efficiencies are summarized in **Table 1**. Purified peroxidase migrated in SDS-PAGE as a single band corresponding to a molecular weight of 48.2 ± 3.0 kDa [43]. The retention time of the elution of protein from the size-exclusion column indicates that the enzyme forms dimers in solution with approximate molecular weight of 92 ± 2 kDa [37]. Thin-layer IEF confirmed the

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Table 1. Purification of RPTP.

Procedure	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
1 Homogenate	9980	68,862	1,297,422	18.84	1	100
2 PEG + $(NH_4)_2SO_4$	5400	11,502	1,293,635	112.47	6	99.7
3 Phenyl-Sepharose	383	498	845,825	1698.78	90	65.2
4 DEAE-Toyopearl	99.5	158	581,665	3676.65	195	44.8
5 Superdex 200	42	67	438,626	6568.22	350	33.8

apparent homogeneity of the protein and enabled us to estimate the pI of the protein to be around 5.4 ± 0.1 [43].

3.2. Steady-State Rate Equation and Kinetic Parameters of RPTP-Catalyzed Oxidation Reaction

In a two-substrate enzyme system, two general mechanisms are possible for the substrate-enzyme interaction: namely, a sequential mechanism or a Ping-Pong mechanism. In the former, both substrates combine with the enzyme to form a ternary complex before catalysis occurs. The substrates can combine with the enzyme either in a random fashion (Random Bi Bi) or in an obligatory order (Ordered Bi Bi) to form the ternary complex. The products thus formed can therefore be released in an ordered or random fashion. In a Ping-Pong mechanism, a ternary substrate-enzyme complex is not formed. The first substrate in a Ping-Pong Bi Bi mechanism combines with the enzyme to form a substituted enzyme intermediate, with the ensuing release of the first product. The second substrate then interacts with the substituted enzyme intermediate to form the second product and regenerate the native enzyme. Ping-Pong and sequential mechanisms can be differentiated by steady-state kinetic analysis of the reaction using the procedures described by Cleland [45,46].

The kinetic mechanism of the H_2O_2-assisted RPTP-catalyzed oxidation of AH_2 reducing substrates was investigated using initial-rate measurements, in which the concentrations of both substrates, -H_2O_2 and AH_2- were varied systematically and the results were analyzed assuming steady-state conditions. The initial rates, v, as a function of hydrogen peroxide or the AH_2 concentration, were fitted to the Michaelis-Menten rate equation (**Eq.A2** of the Appendix) by an iterative process [47].

At pH 4.0, double-reciprocal plots of initial steady-state rates of ferulic acid oxidation *vs.* hydrogen peroxide concentration (0.1 - 2.4 mM), at fixed reducing substrate ferulic acid concentrations, afforded a set of approximately parallel lines, as shown in **Figure 1**. A similar graphic behaviour of the double-reciprocal plots of the data was obtained upon studying the effect of ferulic acid concentrations on the initial rates of the oxidation reaction at different fixed H_2O_2 concentrations (data not shown). Similar trends towards parallel lines in double-reciprocal plots were also observed at different pHs for guaiacol, catechol, ABTS, o-dianisidine and o-phenylendiamine. The obtained trends towards such linear parallel plots point to a Ping-Pong Bi Bi mechanism involving two independent enzyme forms (*i.e.* enzyme forms separated by an irreversible step).

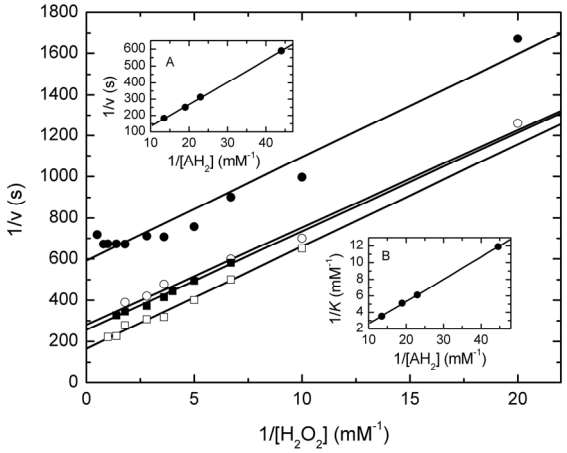

Figure 1. Primary double-reciprocal plot of the initial rate of ferulic acid oxidation as a function of hydrogen peroxide concentration at fixed ferulic acid concentrations (0.023 mM (●), 0.045 mM (○), 0.053 mM (■), 0.076 mM (□)). The insets show the secondary plots of 1/V vs. 1/[ferulic acid] (A) and of 1/K vs. 1/[ferulic acid] (B). See text for other experimental conditions.

Thus, upon representing the intercept (1/V) of the above lines and the inverse of the *K* parameter vs. the reciprocal of the fixed substrate concentration, linear relationships are obtained (insets in **Figure 1** for the ferulic acid case). The values of $K_m^{H_2O_2}$, $K_m^{AH_2}$, V_{max} and k_{cat}, shown in **Table 2**, were calculated from the slopes and intercepts of the corresponding linear fittings of data following **Eqs.A6** and **A7**.

The highest turnover number, k_{cat}, of RPTP was found for the substrate ferulic acid, followed by catechol, ABTS, guaiacol, o-phenylenediamine and o-dianisidine. The highest affinity of the enzyme ($1/K_m$) was for o-dianisidine, followed by ABTS, ferulic acid, o-phenylenediamine, guaiacol and catechol. However, the highest specificity constant, or catalytic efficacy of the enzyme (k_{cat}/K_m), was seen for ferulic acid, followed by ABTS, o-

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Table 2. Kinetic parameters obtained for the H_2O_2-mediated oxidation of substrates by RPTP. See text for experimental conditions.

Substrate	K_m (H_2O_2) (M)	K_m (AH_2) (M)	V_{max} ($M \cdot s^{-1}$)	[E_o] (10^{-10} M)	k_{cat} (s^{-1})	k_{cat}/K_m (H_2O_2) ($M^{-1} \cdot s^{-1}$)	k_{cat}/K_m (AH_2) ($M^{-1} \cdot s^{-1}$)	k_1 ($\mu M^{-1} \cdot s^{-1}$)	k_3 ($\mu M^{-1} \cdot s^{-1}$)
Guaiacol	2.7×10^{-3}	15.2×10^{-3}	1.2×10^{-6}	5.61	2.1×10^{3}	7.8×10^{5}	1.4×10^{5}	0.33	0.08
ABTS	1.8×10^{-3}	4.9×10^{-4}	2.0×10^{-6}	6.05	3.3×10^{3}	1.8×10^{6}	6.7×10^{5}	1.30	4.80
Ferulic acid	1.9×10^{-3}	5.4×10^{-4}	4.8×10^{-6}	8.91	5.4×10^{4}	2.8×10^{7}	1.0×10^{8}	1.82	6.96
o-Dianisidine	7.0×10^{-4}	1.0×10^{-4}	1.2×10^{-7}	2.97	4.0×10^{2}	5.7×10^{5}	4.0×10^{6}	0.25	1.91
o-Phenilendiamine	2.2×10^{-3}	1.4×10^{-3}	7.8×10^{-7}	10.2	7.6×10^{2}	3.5×10^{5}	5.5×10^{5}	0.23	0.26
Catechol	3.5×10^{-3}	8.8×10^{-2}	2.8×10^{-5}	35.0	8.0×10^{3}	2.3×10^{6}	9.0×10^{4}	0.40	0.02

dianisidine, o-phenylenediamine, guaiacol and catechol. Similar reactivities for these substrates have been found for African [30] and *Chamaerops excelsa* [48] palm tree peroxidases.

3.3. Microscopic Rate Constants

Peroxidases catalyze the oxidation of AH_2 organic substrates, using H_2O_2 (or other peroxides) as an electron acceptor in a three-step catalytic cycle involving different intermediate enzyme forms [19,20]:

$$E + H_2O_2 \xrightarrow{k_1} EI + H_2O \quad (1)$$

$$EI + AH_2 \xrightarrow{k_2} EII + AH^{\bullet} \quad (2)$$

$$EII + AH_2 \xrightarrow{k_3} E + H_2O + AH^{\bullet} \quad (3)$$

where E is the native enzyme. The monoelectronic oxidation of the native state E affords an intermediate state termed EI (**Eq.1**). EI is responsible for the oxidation of the electron-donor substrate (AH_2), accepting one proton and one electron and generating its free radical ($AH^{\bullet}$), together with another enzyme state, designated EII (**Eq.2**). Finally, EII is reduced by a second molecule of substrate (**Eq.3**), giving rise to a second free radical ($AH^{\bullet}$). The microscopic constant k_1 (the constant of EI formation) indicates the reactivity of the enzyme towards hydrogen peroxide, and k_3 (the constant of EII reduction) represents the reactivity of the enzyme towards the reducing substrate.

With a view to calculating the microscopic constants (k_1 and k_3) of the oxidation of the substrates by RPTP, the oxidation rates of the substrates were fitted for each concentration of AH_2 and H_2O_2, assuming the steady-state approach and considering that $k_2 > k_3$.

As may be seen in the Appendix, double-reciprocal plots (1/v vs. 1/[H_2O_2]) allowed us to calculate the A and B values for each AH_2 concentration. **Figure 2** shows the plot of A vs. B (**Eq.A8**) for four ferulic acid concentrations. From this straight line it is possible to calculate the value of k_1 (formation constant of Compound I) for peroxidase-mediated ferulic acid oxidation. The value obtained, as well as those obtained for guaiacol, catechol, ABTS, o-dianisidine and o-phenylendiamine, is shown in **Table 2**.

As shown in the Appendix, double reciprocal plots (1/v vs. 1/[AH_2]) allowed us to calculate the A and B values (**Eq.A9**) for each H_2O_2 concentration. **Figure 3** shows the plot of A vs. B values for three H_2O_2 concentrations during the oxidation of ferulic acid. With this plot it is possible to calculate the value of k_3 (the forma- values (**Eq.A9**) for each H_2O_2 concentration. **Figure 3**

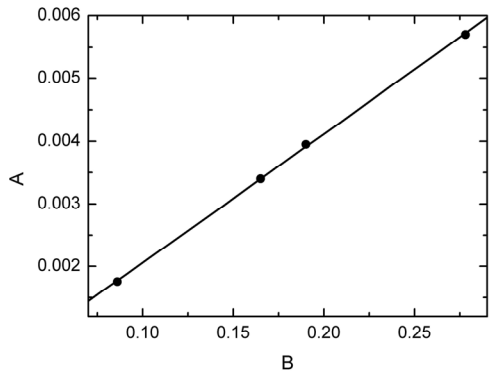

Figure 2. Secondary plot of parameters A ($nmol \cdot s^{-1}$) vs. B (mM) obtained by varying H_2O_2 concentra- tions for four ferulic acid concentrations (0.023, 0.045, 0.053, 0.076 mM). See text for other expe- rimental conditions.

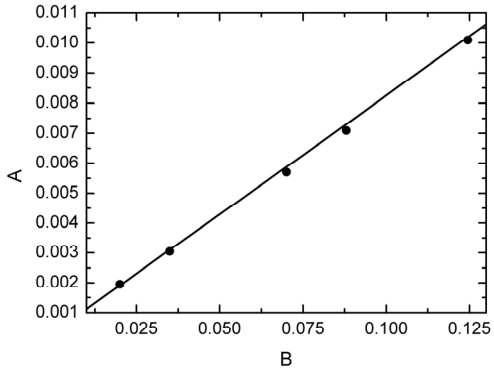

Figure 3. Secondary plot of parameters A ($nmol \cdot s^{-1}$) vs. B (mM) obtained by varying ferulic acid con- centrations for five H_2O_2 concentrations (0.1, 0.15, 0.30, 0.50, 1.00 mM). See text for other experi- mental conditions.

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shows the plot of A vs. B values for three H_2O_2 concentrations during the oxidation of ferulic acid. With this plot it is possible to calculate the value of k_3 (the formation constant of Compound II) for peroxidase-mediated ferulic acid oxidation. The value obtained, as well as those obtained for guaiacol, catechol, ABTS, o-dianisidine and o-phenylendiamine, is also listed in **Table 2**.

These A vs. B plots allowed us to calculate the real reaction constants (k_i) from steady-state measurements of the oxidation rate, avoiding their dependence on the substrate concentration [20].

From the rate constant values (k_i) shown in **Table 2**, it may be deduced that the RPTP is capable of oxidizing phenolic and aromatic amine substrates. The data obtained show that the most reactive substrate for RPTP was ferulic acid, followed by ABTS, the aromatic amines o-dianisidine and o-phenylendiamine, guaiacol and catechol being the least reactive substrates. The high reactivity of ABTS must be due to its greater number of H-bond acceptor atoms, because the highest values of reactivity constants are seen for substrates with the most acceptor H-bonds (www.chemicalregister.com). ABTS has ten H-bond acceptor sites; ferulic acid and o-dianisidine four, and o-phenylendiamine, guaiacol and catechol two. In respect of H-bond donor sites—ABTS, ferulic acid, o-dianisidine, o-phenylendiamine, catechol—each has two H-bond donor sites and guaiacol only one.

Similar studies addressing kinetic parameters and microscopic rate constants carried out with African [30] and *Chamaerops excelsa* [48] palm tree peroxidases have shown that these enzymes exhibit greater reactivity towards ferulic acid and ABTS, followed by the aromatic amines o-dianisidine, o-phenylendiamine and, finally, by phenolic substrates with one or two hydroxyl groups in their chemical structures. In contrast, both soybean and peanut peroxidases are more reactive towards guaiacol than towards amines [30,49]. Horseradish and tobacco peroxidases have been reported to be equally reactive towards guaiacol and o-dianisidine and about 10 - 15 times less reactive towards o-phenylendiamine [43].

Commonly substrate specificity studies of peroxidases are conducted with only one substrate present, apart from H_2O_2, in the reaction mixture at a given time; *i.e.* without any alternative substrates able to undergo the same reaction. This is because the presence of competing substrates tends to complicate the analysis, without providing much more information than would be obtained by studying the substrates separately. However, this implies an important difference between experimental practice and the physiological conditions under which enzymes usually exist. Thus, most enzymes are not perfectly specific for a single substrate and must often select between several that are available simultaneously. Therefore, to be physiologically meaningful enzyme specificity must be defined in terms of how well the enzyme can discriminate between the substrates present in the same reaction mixture. This does not mean that it cannot be determined from the kinetic parameters of the enzyme for separate substrates, but it does mean that these parameters need to be interpreted correctly and not on a casual basis [50].

3.4. Substrate Inhibition

In order to further check the kinetic mechanism of the substrate oxidation reactions catalyzed by RPTP, inhibition studies were carried out. One of the characteristic features of Ping-Pong reaction mechanisms is the occurrence of competitive substrate inhibition by both substrates [29].

In the Ping-Pong reaction mechanism, since the three forms of the enzyme—E, CoI and CoII—are so similar, it is reasonable to expect AH_2 to have some affinity for E as well as CoI and CoII and, if the active sites in CoI and CoII are not too full for the adsorption of H_2O_2, for H_2O_2 to show some affinity for CoI and CoII [51]. In peroxidases, reports have been made of the formation of a non-productive, or dead-end, complex between AH_2 and E and the reaction of high concentrations of H_2O_2 with CoI, affording H_2O_2 and O_2 [52].

As stated in the Appendix, following the reciprocal of **Eq.A13**, plots of 1/v vs. $1/[H_2O_2]$ at fixed $[AH_2]$ should be linear and should intersect on the y axis. This graphical behaviour was observed at fixed ferulic acid inhibitory concentrations (**Figure 4**).

In this sense, **Figure 4** (inset) shows this linear plot for ferulic acid. For the other substrates studied, a similar

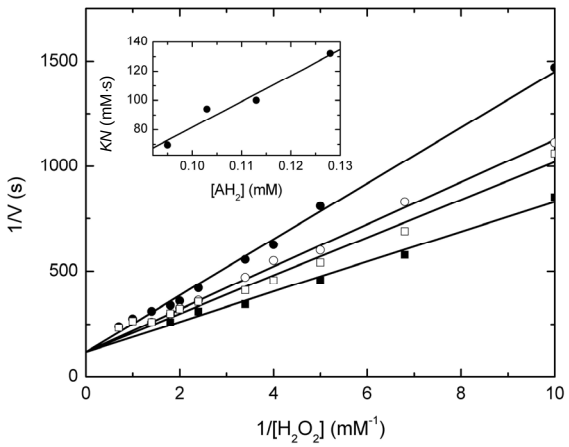

Figure 4. Primary double-reciprocal plot of the initial rate of ferulic acid oxidation as a function of hydrogen peroxide concentration at fixed ferulic acid inhibitory concentrations (0.095 mM (■), 0.104 mM (□), 0.113 mM (○), 0.128 mM (●)). The inset shows the secondary plot of K/V vs. ferulic acid concentration. See text for other experimental conditions.

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degree of substrate competitive inhibition was found (data not shown).

Alternatively, at fixed inhibitory values of H_2O_2 concentration, the v vs. $[AH_2]$ data analysis provided a series of alternative equations similar to **Eqs.A13-A16**.

Accordingly, the corresponding plots of 1/v vs. $1/[AH_2]$ at fixed $[H_2O_2]$ are also linear and intersect on the y axis and the plots of *K*/V vs. $[H_2O_2]$ should also be linear. This graphic behaviour was obtained for ferulic acid at fixed $[H_2O_2]$ (**Figure 5**) and also for the rest of substrates studied (data not shown).

Thus, competitive substrate inhibition was observed in the H_2O_2-assisted RPTP-catalyzed oxidation reactions for both substrates, as would be expected for a Ping-Pong reaction mechanism. The corresponding inhibition constants— $K_{SI}^{H_2O_2}$ and $K_{SI}^{AH_2}$ —obtained, for the case of ferulic acid, were 1.24×10^{-3} M and 2.7×10^{-5} M, respectively.

In light of the above, the combined initial-rate and substrate inhibition results exclude an ordered or random sequential reaction mechanism, and are only consistent with a Ping-Pong Bi Bi mechanism according to the notation of Cleland [29] as the minimal kinetic model for H_2O_2-assisted RPTP-catalyzed substrate oxidation reactions.

Ping-Pong reaction kinetics has also been observed for several other peroxidase-catalyzed oxidations mediated by Compound I. The results of initial-rate studies of the hydrogen peroxide-supported oxidation of guaiacol by turnip peroxidase [53] and of the oxidation of ferrocytochrome c catalyzed by horseradish peroxidase [54] and yeast cytochrome c peroxidase [55] are consistent with a Ping-Pong mechanism. Initial-rate kinetic studies of the oxidation of ferrocytochrome c by *Pseudomonas aeruginosa* cytochrome c peroxidase yielded intersecting plots, which were initially interpreted as indicating a sequential reaction mechanism [56]. However, subsequent studies demonstrated that the intersecting plots arose from the formation of an inactive hydrogen peroxide-enzyme complex, and the mechanism of the reaction was reinterpreted to be of the modified Ping-Pong type [57]. It has recently been reported that *Chamaerops excelsa* palm tree peroxidase also exhibits a Ping-Pong Bi Bi mechanism for the H_2O_2-assisted catalyzed oxidation reactions of guaiacol, ABTS, o-dianisidine and o-phenylendiamine [48].

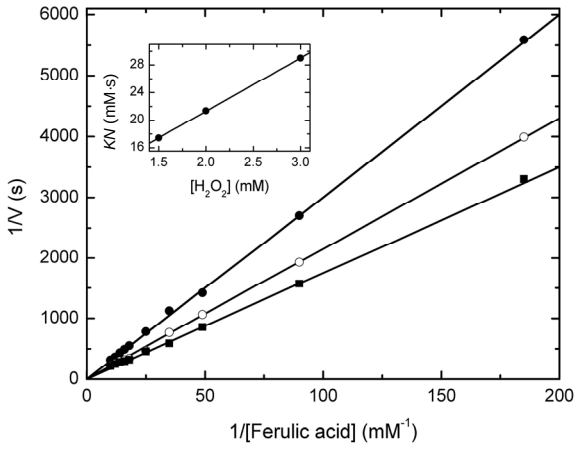

Figure 5. Primary double-reciprocal plot of the initial rate of ferulic acid oxidation as a function of ferulic acid concentration at fixed hydrogen peroxide inhibitory concentrations (1.5 mM (■), 2.0 mM (○), 3.0 mM (●)). The inset shows the secondary plot of K/V vs. H_2O_2 concentration. See text for other experimental conditions.

3.5. Partitioning Ratio (r) for the Inactivation of RPTP by H_2O_2

Using plots of the percent residual activity for each substrate against the [peroxide]/[enzyme] ratio, the ratio required in each case for 100% inactivation can be obtained from the intercept of the fitted line. From this value, the partitioning ratio can be calculated using the following equation:

$$A_R = \frac{A_t}{A_o} = 1 - \frac{1}{1+r}\frac{[H_2O_2]}{[RPTP]} \quad (4)$$

where A_R is the residual activity; A_t and A_o are the activities at time (t) (end of the reaction) and zero respectively; (r) is the partitioning ratio or inactivation turnover number (number of catalytic cycles given by enzymes before their inactivation), and $[H_2O_2]$ and [RPTP] are the initial concentrations of H_2O_2 and enzyme [33].

Figure 6 shows the plots of the percent residual activity against the $[H_2O_2]$/[RPTP] ratio for the guaiacol substrate. In light of the consumption of two moles of H_2O_2 in each catalytic cycle (one mole for the formation of Compound I and another for inactivation or catalysis) [58-60], the (r) value, calculated from the corresponding fitting of the data to the first linear section of the curve (**Figure 6**), was 2154 ± 100.

3.6. Kinetics of Inactivation by H_2O_2

In the absence of reducing substrate, the class I, II and III peroxidases studied to date undergo suicide inactivation by H_2O_2. The inactivation kinetics shows profiles that differ for each enzyme. The inactivation kinetics of RPTP by hydrogen peroxide, at a range of different concentrations, using guaiacol as co-substrate, is biphasic, with a rapid inactivation step in the first 10 - 15 min, followed by a longer-lasting, slow step (data not shown). The same biphasic behaviour has been reported for the inactivation by H_2O_2 of HRP [32,33,61] and melon peroxidase [62]. Other peroxidases studied to date also

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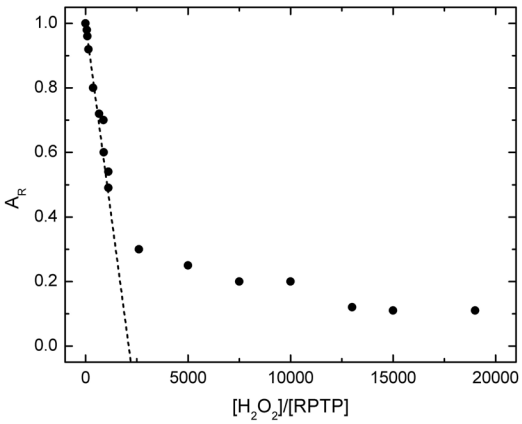

Figure 6. Sensitivity to inactivation of RPTP at different $[H_2O_2]/[RPT]$ molar ratios. RPTP was incubated with molar excesses of peroxide in universal buffer (30 mM, pH 6.5) and when the reaction was complete (24 h incubation), the percentage residual activities were measured with guaiacol at a fixed concentration of the enzyme (136 nM).

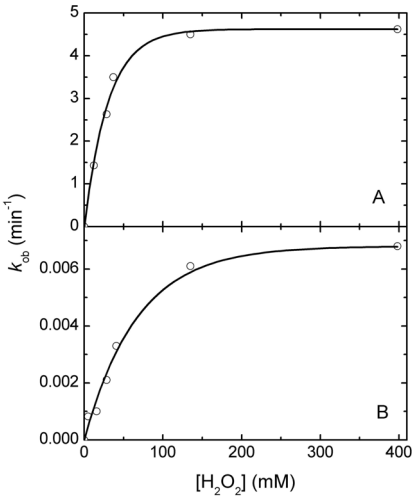

Figure 7. Biphasic inactivation kinetics of RPTP by H_2O_2. Plot of k_{ob} (first-order inactivation rate constant) against H_2O_2 concentration for the first and rapid inactivation step (A) and for the longer-lasting slow step (B).

exhibit different types of behaviour [33,48].

Then, the biphasic behaviour of data of residual peroxidase activity *versus* time could be fitted to a sum of exponentials:

$$A_R = a \cdot e^{-k_{ob}t} + b \cdot e^{-k_{ob'}t} \quad (5)$$

Consequently, following **Eq.6**, a plot of the logarithms of the percentage residual activities ($\ln A_R$) against time afforded straight lines for each segment of time (data not shown), with slopes equivalent to the observed rate constants of the inactivation (k_{ob}, $k_{ob'}$) [38].

$$\ln \frac{[A_t]}{[A_o]} = -k_{ob}t \quad (6)$$

The inactivation of RPTP by H_2O_2 clearly showed saturation kinetics, as seen from the hyperbolic curves fitted to the plot of k_{ob} against $[H_2O_2]$ for each segment of time (**Figure 7**).

Then, the observed first-order rate constants of inactivation (k_{ob}) can be fitted to the following equation:

$$k_{ob} = \frac{k_{inact}^{app}[H_2O_2]}{K_I^{app} + [H_2O_2]} \quad (7)$$

where k_{inact}^{app}, a first-order inactivation rate constant, and K_I^{app}, an inhibitor-binding constant, were obtained from the corresponding fitting of the data by a linear regression model [63].

In the absence of reducing substrates and at high H_2O_2 concentrations, RPTP is inactivated in a time- and H_2O_2 concentration-dependent process, exhibiting suicide or mechanism-based inactivation kinetics. Similar types of behaviour have been reported by other authors [16,32,33, 61,64] in studies addressing H_2O_2-mediated inactivation under identical experimental conditions of temperature, pH, and the concentrations of peroxidase and H_2O_2. Nevertheless there are, differences in the shapes of the inactivation curves, in the magnitudes and rates of inactivation at specific H_2O_2 concentrations, and in the maximum rate of inactivation (k_{inact}^{app}) as compared with the results reported here.

The inactivation process involves the participation of two pathways, one reversible and other irreversible, which may or may not function independently of each other and whose individual contribution to the overall inactivation process seems to be dependent upon the H_2O_2 concentration. For enzyme inactivation, a second molecule of hydrogen peroxide would be required; not for the inactivation *per se* but for the formation of Compound III. Once Compound III has been formed, a fraction of that population would be transformed into an inactive species. Another interpretation would be that once a molecule of Compound III has been formed, it has a certain probability of decaying into an inactive species instead of decaying into an active one that would again become engaged in the catalytic cycle.

In the case of Compound I, several authors [32,33,64] have suggested the existence of a partitioning between the two pathways. Their model, based on studies at high H_2O_2 concentrations, invokes a further partitioning between these two inactivation pathways and suggests the existence of a catalytic reaction in which H_2O_2 would be consumed with relatively little harm to the enzyme [64].

The model is actually a simplification of the full kinetic approach developed previously by Arnao *et al.*,

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Table 3. Apparent kinetic constants calculated for the inactivation of RPTP (*, #) and CEP variants by H_2O_2 at 25˚C.

Substrate	r	k_{inact}^{app} (s^{-1})	K_I^{app} (mM)	$1/K_I^{app}$ (M^{-1})	k_{cat}^{app} (s^{-1})	k_{cat}^{app}/K_I^{app} ($s^{-1}\cdot M^{-1}$)	$k_{inact}^{app}/K_I^{app}$ ($s^{-1}\cdot M^{-1}$)
Guaiacol*	2154	0.086	25.63	0.039	185.2	723	3.36
Guaiacol#	2154	0.0083	66.03	0.015	17.9	270	1.26
ABTS	4844	3.24	845	1.18	15694	18573	3.83
Guaiacol	3711	3.85	204	4.90	14287	70034	18.87
o-Dianisidine	951	1.27	39.6	25.2	1209	30530	32.07
o-Phenilendiamine	2147	1.79	87.1	11.5	3839	44076	20.55

*Rapid inactivation of RPTP. #Slow inactivation of RPTP.

[63], although it does offer a reasonable approximation that would be suitable for comparative purposes, as required here. Double-reciprocal plots of rate (or k_{ob}) of inhibition vs. inhibitor concentration data are often used to explore the formation of complexes between inhibitors and enzymes [64-66]. Here we used these linear least-squares fits of the data (data not shown) to determine the relationship between the different enzymes and H_2O_2. For each variant we obtained the apparent values of the inactivation rate constant (k_{inact}^{app}) and the dissociation constant (K_I^{app}) of the [Compound I.H_2O_2] complex and, together with the already determined inactivation turnover numbers (r) (see above), the values of the catalytic rate constant (k_{cat}^{app}), using the relationship:

$$r = \frac{k_{cat}^{app}}{k_{inact}^{app}} \quad (8)$$

These findings, together with the corresponding to the affinity of the enzyme for the inhibitor ($1/K_I^{app}$) and to the efficiencies of catalysis (k_{cat}^{app}/K_I^{app}) and inactivation ($k_{inact}^{app}/K_I^{app}$), are summarized in **Table 3**, which—for comparative purposes—also includes the results of the inactivation of *Chamaerops excelsa* peroxidase [40]. CEP is the most active of all currently known peroxidases, suggesting that the active site of the enzyme has evolved not only to improve catalytic efficiency but also to prevent inactivation by the highly reactive H_2O_2 substrate.

The accessibility of the substrate to the reducing binding site in the haem pocket and its affinity for the product are important for enzyme activity. Substrate specificity may be modified by changes that affect the reducing binding site [67] and it may be possible to further adjust the specificity and level of activity of RPTP by judicious changes in this region. The glycosylation of RPTP also appears to be significant in protecting the enzyme from inactivation.

RPTP shows good resistance to inactivation by hydrogen peroxide, opening the possibility of further studies related to the mechanisms of exchange of hydrogen peroxide in peroxidases and suggesting, as in other studies [17], that RPTP acts as a very robust enzyme.

4. CONCLUSIONS

As results of the analysis of the initial rates *vs.* H_2O_2 and reducing substrate concentrations carried out, the proposed steady-state kinetic model of the H_2O_2-supported oxidation of different organic substrates by RPTP is a substrate-inhibited Ping-Pong Bi Bi reaction mechanism. The phenomenological approach used expresses the peroxidase Ping-Pong mechanism in the form of the Michaelis-Menten equation and affords an interpretation of the effects in terms of the kinetic parameters $K_m^{H_2O_2}$, $K_m^{AH_2}$, k_{cat}, $K_{SI}^{H_2O_2}$, $K_{SI}^{AH_2}$ and of the microscopic rate constants k_1 and k_3 of the shared three-step peroxidase catalytic cycle. This substrate-inhibited Ping-Pong Bi Bi reaction mechanism has been also proposed for peroxidase from *Chamaerops excelsa* palm tree [48].

The kinetics of inactivation of RPTP in the oxidation of guaiacol by hydrogen peroxide shows suicide inactivation behavior similar to that of most classical peroxidases [32,33,38,41]. The model used in these experiments [38] provides satisfactory parameters for the inactivation kinetics by hydrogen peroxide, showing the high capacity of the enzyme to act the substrate at a turnover of molecules up to 2154 and exhibits an apparent rate constant of catalysis of 185 s^{-1} and 18 s^{-1}. These values may indicate that RPTP exhibits a good performance against inactivation by hydrogen peroxide, opening the possibility of further studies related to the mechanisms of exchange of hydrogen peroxide in peroxidases and pointing, besides other studies [17], that RPTP acts as a very robust enzyme.

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APPENDIX

The rate equation for an enzyme-catalyzed Ping-Pong reaction with two substrates, H_2O_2 and AH_2, in the absence of products and at non-inhibitory substrate concentrations, is given by

$$v = \frac{V_{max}[H_2O_2][AH_2]}{K_m^{H_2O_2}[AH_2] + K_m^{AH_2}[H_2O_2] + [H_2O_2][AH_2]} \quad (A1)$$

which can be cast in the form of a rectangular hyperbola for fixed values of $[AH_2]$:

$$v = \frac{V[H_2O_2]}{K + [H_2O_2]} \quad (A2)$$

where V and *K* parameters are as follows:

$$V = \frac{V_{max}}{1 + K_m^{AH_2}/[AH_2]} \quad (A3)$$

$$K = \frac{K_m^{H_2O_2}}{1 + K_m^{AH_2}/[AH_2]} \quad (A4)$$

Thus plots of 1/v vs. $1/[H_2O_2]$ are linear and parallel at different fixed $[AH_2]$ since:

$$\frac{1}{v} = \frac{1}{V} + \frac{K}{V[H_2O_2]} \quad (A5)$$

Furthermore, the reciprocals of **Eqs.A3** and **A4** are

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given by:

$$\frac{1}{V}=\frac{1}{V_{max}}+\frac{K_m^{AH_2}}{V_{max}}\frac{1}{[AH_2]} \quad (A6)$$

$$\frac{1}{K}=\frac{1}{K_m^{H_2O_2}}+\frac{K_m^{AH_2}}{K_m^{H_2O_2}}\frac{1}{[AH_2]} \quad (A7)$$

For non-inhibitory concentrations of H_2O_2, the initial rates of the substrate oxidation by peroxidases, following the three-step catalytic cycle [19,20], can be fitted to the following equation:

$$v=\frac{A[H_2O_2]}{B+[H_2O_2]} \quad (A8)$$

where A = 2[E]k_3[AH_2] and B = (k_3/k_1)[AH_2]. Double-reciprocal plots (1/v vs. 1/[H_2O_2]) allowed us to calculate A and B values for each AH_2 concentration

Similarly, the dependence of v on [AH_2] may be written as:

$$v=\frac{A[AH_2]}{B+[AH_2]} \quad (A9)$$

where A = 2[E]k_1[H_2O_2] and B = (k_1/k_3)[H_2O_2]. Double reciprocal plots (1/v vs. 1/[AH_2]) allowed us to calculate the A and B values for each H_2O_2 concentration

From **Eq.A8**, the catalytic efficacy for the utilization of H_2O_2 would be given by:

$$\frac{k_{cat}}{K_m^{H_2O_2}}=\frac{2k_3[AH_2]}{(k_3/k_1)[AH_2]}=2k_1 \quad (A10)$$

while the catalytic efficacy for the utilization of the substrate AH_2 would be given by:

$$\frac{k_{cat}}{K_m^{AH_2}}=\frac{2k_1[H_2O_2]}{(k_1/k_3)[H_2O_2]}=2k_3 \quad (A11)$$

Consequently, the reactivity of the enzyme with hydrogen peroxide is determined by the value of the constant k_1. However, its reactivity with the reducing substrate is determined by the constant k_3.

The occurrence of competitive substrate inhibition by both substrates in the reaction mechanism means that in the denominator of the rate **Eq.A1**, the $K_m^{H_2O_2}[AH_2]$ term can be multiplied by $\left(1+[AH_2]/K_{IS}^{AH_2}\right)$ and the $K_m^{AH_2}[H_2O_2]$ term by $\left(1+[H_2O_2]/K_{IS}^{H_2O_2}\right)$ where $K_{IS}^{AH_2}$ and $K_{IS}^{H_2O_2}$ are the dissociation constants of AH_2 from EAH_2 and of H_2O_2 from CoI H_2O_2 and/or CoII H_2O_2 complexes, respectively, (**Eq.A12**), and double competitive substrate inhibition would be exhibited when AH_2 and H_2O_2 are varied. Thus, the corresponding rate equation would be **Eq.A12**.

At fixed inhibitory values of the AH2 concentration, the v vs. [H_2O_2] data were fitted to the following rate equation of competitive inhibition:

$$v=\frac{V_{max}^{H_2O_2}[H_2O_2]}{K_m^{H_2O_2}\left(1+\frac{[AH_2]}{K_{SI}^{AH_2}}\right)+[H_2O_2]} \quad (A13)$$

For competitive inhibition, the *K* and V of **Eq.A2** are defined by

$$V=\frac{V_{max}}{1+K_m^{AH_2}/[AH_2]} \quad (A14)$$

$$K=\frac{K_m^{H_2O_2}\left(1+[AH_2]/K_{SI}^{AH_2}\right)}{1+K_m^{AH_2}/[AH_2]} \quad (A15)$$

Thus, following the reciprocal of **Eq.A13**, plots of 1/v vs. 1/[H_2O_2] at fixed [AH_2] should be linear and should intersect on the y axis.

Furthermore, the plots of *K*/V vs. [AH_2] should be linear because

$$\frac{K}{V}=\frac{K_m^{H_2O_2}}{V}+\frac{K_m^{H_2O_2}[AH_2]}{K_{SI}^{AH_2}V_{max}} \quad (A16)$$

Alternatively, at fixed inhibitory values of H_2O_2 concentration, the v vs. [AH_2] data analysis should provide a series of alternative equations similar to **Eqs.A13-A16**.

$$v=\frac{V_{max}[H_2O_2][AH_2]}{K_m^{H_2O_2}[AH_2]\left(1+[AH_2]/K_{SI}^{AH_2}\right)+K_m^{AH_2}[H_2O_2]\left(1+[H_2O_2]/K_{SI}^{H_2O_2}\right)+[H_2O_2][AH_2]} \quad (A12)$$

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