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Short communication

Substrate ionization energy influences the epoxidation of *m*-substituted styrenes catalyzed by chloroperoxidase from *Caldariomyces fumago*

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ABSTRACT

Chloroperoxidase from the fungus *Caldariomyces fumago* is a versatile heme-peroxidase, which is able to catalyze olefin epoxidation. In this report, the epoxidation of *m*-substituted styrene catalyzed by chloroperoxidase was studied. The catalytic data fit the Hill's model, and the activity rate (k_{cat}) constants were strongly dependent of nature of substituents. The epoxidation k_{cat} values varied as follows: *m*-aminostyrene > styrene > *m*-methylstyrene > *m*-chlorostyrene > *m*-nitrostyrene. The catalytic rate constant for *m*-aminostyrene, an electron-releasing substituent, was 298 times higher than these for *m*-nitrostyrene, an electron-withdrawing compound. These results were quantitatively analyzed and a good linear correlation between the experimental catalytic constants of *m*-substituted styrenes and their ionization energies, calculated at B3LYP level of theory, was demonstrated.

1. Introduction

Chloroperoxidase from the fungus *Caldariomyces fumago* (CPO; E.C. 1.11.1.10) is a versatile heme-peroxidase that uses hydrogen peroxide and halide ions for halogenation reactions of a variety of organic substrates. In addition, of halogenation reactions, CPO is able to catalyze different reactions including hydrogen peroxide dismutation, sulfide and alcohol oxidations, benzylic and allylic hydroxylation, and olefin epoxidations [1,2]. This enzyme catalyzes the epoxidation of a variety of olefin substrates with high yields, and several strategies have been employed in order to enhance its catalytic performance [3–6]. CPO-mediated catalytic epoxidation shows several advantages because this enzyme is the fastest peroxidase known so far, it does not need cofactors, and the final electron acceptor is the environment friendly compound hydrogen peroxide. Moreover, the enzyme can be produced in large quantities and it is stable to storage conditions. CPO-mediated epoxidation of olefins has been objet of intensive research due to the importance of chiral epoxides in pharmaceutical and fine chemical industries [7,8].

The catalytic cycle of CPO peroxidase-like oxidations, using hydrogen peroxide or suitable alkyl peroxides, start with the formation of the highly reactive intermediate iron(IV)-oxo radical, named Compound I (Cpd I) [9]. Subsequently, the reaction proceeds in a stepwise manner with the C–O bond formation on the organic substrate, followed by C–O–C ring formation. The ring formation step is barrier-less for the low spin pathway and the C–O bond formation is the rate determining step [10,11].

The correlation between the ionization potential of substrate and the enzymatic transformation rate has been reported for peroxidases [12–14] and as well for CPO in the oxidation of organosulfur compounds [15]. Recently, the rate constants (k_{cat}) of CPO-catalyzed oxidation on a variety of substrates have been reported [16]. In particular, for *p*-substituted styrene derivatives a linear Hammett relationship between log k_{catX}/k_{catH} and σ-Hammett constants have been found. Linear free energy relationship has also been established in the oxidation of styrene derivatives catalyzed by divers biomimetic porphyrin compounds [17]. Moreover, epoxidations of olefin substrates by these biomimetic iron(IV)-oxo porphyrin compounds has been extensively studied during the last decades through experimental and theoretical approaches [18,19]. Sainna et al. [20] have performed a comprehensive study on the epoxidation of alkenes using biomimetic iron(IV)-oxo porphyrin models. These authors have shown that the experimental rate constants are correlated with the ionization energies of olefin substrates. This correlation has been previously established on theoretical grounds using Density Functional Theory (DFT) calculations for the epoxidation of a variety of olefin substrates mediated by Cpd I model [21]. In order to evaluate if this correlation is also observed in the CPO-catalyzed epoxidations, the enzymatic reaction kinetics with divers *m*-substituted styrenes were determined and correlated with the substrate ionization energies calculated at DFT level.

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2. Materials and methods

2.1. Chemicals

Hydrogen peroxide (30% *w*/*w*) and 2-methyl-2-propanol (2M2P) were obtained from Merck KGaA (Darmstadt, Germany). Styrene and *m*-substituted derivatives were purchased from Sigma-Aldrich (St. Louis, MO). Chloroperoxidase from *C. fumago* was obtained and purified as previously reported [22]. The enzyme preparation contained 6530 U mL^{-1}, with a $R_Z = 1.0$ and an enzyme concentration of 289 μmol L^{-1}. All other chemicals, unless otherwise stated, were supplied by Sigma-Aldrich.

2.2. Enzyme activity and determination of catalytic parameters of CPO

Peroxidase activity of CPO was determined spectrophotometrically using guaiacol as substrate at pH 6.0 and monitoring the absorbance increase at 470 nm. The transformation rate was estimated by using an extinction coefficient of 26,600 M^{-1} cm^{-1}, as guaiacol is converted to tetraguaiacol [23].

Epoxidation reactions were performed in a 60 mM phosphate buffer, pH 6.0, containing substrate (0.10 to 10.0 mM), 15% (*v*/*v*) co-solvent (2M2P) and 800 nM CPO final concentration. The reaction was started by adding hydrogen peroxide solution (40 mM) at a rate of 0.2 μmol/min flow until reach 1 mM of final concentration (saturating concentration). The reactions were stopped after 5 min by adding 1 mL acetonitrile and rapid cooling in ice/water bath. Then, the reaction mixtures were analyzed in a Knauer high-resolution liquid chromatography (HPLC) equipped with *Smartline* 2850 Photodiode Array Detector (HPLC-PDA). The elution was performed with a mobile phase of 65:35 (*v*/v) acetonitrile-water, with a flow of 1.0 mL min^{-1}, through a reverse phase C_{18} column 5 μm Eurospher 100–5 (250 × 4.6 mm). The detection wavelengths were set at 207 nm and 216 nm to measure the generated diols and epoxides, respectively. All extracts obtained from different experiments were filtered using 0.22 μm nylon syringe filters (Membrane Solutions), prior to their analysis by HPLC. Enzyme assays and other UV–VIS experiments were performed with an UV–VIS spectrophotometer UV2310II (Techcomp).

Prior to catalytic tests, calibration curves for each substrate and their corresponding epoxides were obtained. Hill's equation (Eq. (1)) was used as adjustment model to determine the catalytic parameters of CPO-catalyzed epoxidation of styrene derivatives [24]

$$\frac{v}{E_t} = k_{cat}\left(\frac{[S]^n}{K' + [S]^n}\right) \quad (1)$$

where *v* is the reaction rate calculated as total concentration produced of epoxides per minute, E_t is the total concentration of enzyme and k_{cat} represents the catalytic rate constant. The initial concentration of substrate is defined by [*S*], *n* corresponds to the substrate cooperativity to the enzyme and *K*′ is a constant including different interaction factors and the intrinsic dissociation constant K_S of enzyme-substrate complex. CPO specific activity is defined as v/E_t ratio, expressed in min^{-1} units.

2.3. Computational methods

DFT calculations were performed at UB3LYP/6–311 + g(2d,p) level of theory [25] for all styrene substrates. Geometry optimizations were carried out without symmetry restrictions for both neutral and ionic species. Ionization energies (IE) were evaluated as the energy difference between the radical-cation and neutral species, according the following equation: $S \rightarrow S^+ + e^-$ since has been demonstrated that B3LYP functional give precise estimations for both electronic and energetic properties of organic compounds [26].

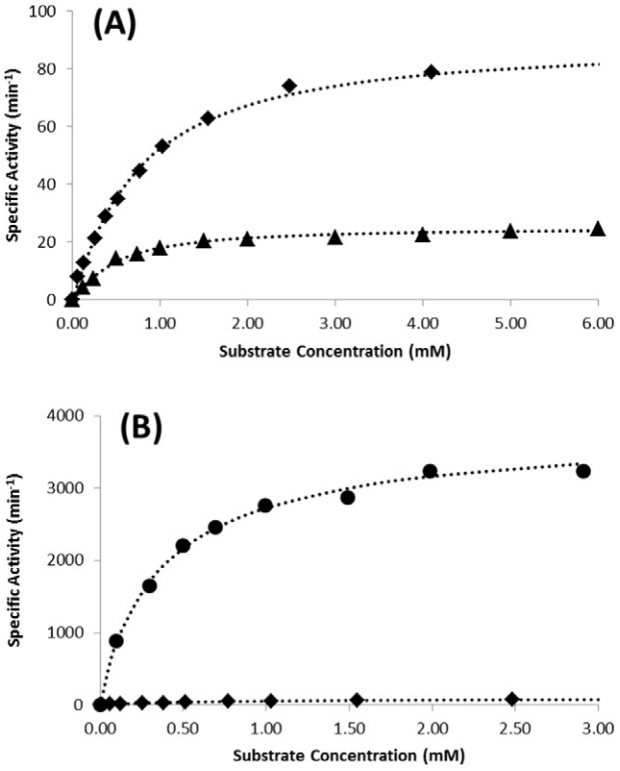

Fig. 1. Specific activity of CPO for the epoxidation of styrene (♦), *m*-aminostyrene (▲) and *m*-chlorostyrene (●) derivatives. The pointed lines correspond to the fit of experimental data to Hill's equation.

3. Results and discussion

The enzymatic activity of CPO in the epoxidation of *m*-subtituted styrenes was evaluated at pH 6.0. It is well known, that CPO is more active as halogenase at low pH, while the peroxidase activity is favored at pH 6.0 [27]. In most of cases, the kinetic data fitted a sigmoidal behavior as can be observed in the Fig. 1. Styrene derivatives with electron-withdrawing substituent showed lower catalytic activities than those derivatives with electron-donor substituents (Fig.1), as in the cases of *m*-nitro and *m*-amino styrene derivatives, respectively. The experimental data fit the Hill equation from which the catalytic constants were determined (Table 1). The substrate cooperativity to the enzyme (*n*) and the "substrate affinity" (*K*′) are also shown in Table 1. In the cases where *n* was equal to 1.0, the Hill equation becomes the rectangular hyperbolic equation and therefore *K*′ became the Michaelis–Menten constant (K_m) corresponding to the substrate concentration that yields half-maximal velocity.

The nitro derivative showed the lower catalytic constant (13 min^{-1}), whereas the amino derivative showed the highest k_{cat} value (3869 min^{-1}). A deeper quantitative analysis was performed. Initially, the kinetic data was correlated with σ-Hamett constant, since previous report has showed a linear correlation between σ constant

Table 1
Catalytic parameters of CPO epoxidation of *m*-substituted styrenes obtained from Hill's equation and substrate ionization energies (IE) calculated at UB3LYP/6–311(2d,p) level of theory.

m-Substituent	k_{cat} (min^{-1})	*K*′ (Moln L^{-n})	*n*	Hill model adjustment (R^2)	IE (eV)
H	90 ± 6	0.7 ± 0.2	1.1 ± 0.1	0.999	8.159
NO_2	13 ± 2	6.7 ± 1.2	1.2 ± 0.2	0.994	8.762
Cl	25 ± 1	0.39 ± 0.04	1.2 ± 0.2	0.981	8.302
CH_3	58 ± 1	0.26 ± 0.03	2.0 ± 0.2	0.985	7.974
NH_2	3869 ± 309	0.42 ± 0.08	0.9 ± 0.1	0.992	7.109