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Metalloporphyrins as cytochrome P450 models for chlorhexidine metabolite prediction

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ABSTRACT

The catalytic oxidation of chlorhexidine (CHX, a strong microbicidal agent) mediated by ironporphyrins has been investigated by using hydrogen peroxide, *m*CPBA, *t*BuOOH, or NaOCl as oxidant. All of these oxygen donors yielded *p*-chloroaniline (pCA) as the main product. The higher pCA yields amounted to 71% in the following conditions: catalyst/oxidant/substrate molar ratio of 1:150:50, aqueous medium, FeTMPyP as catalyst. The medium pH also had a strong effect on the pCA yields; in physiological pH, formation of this product was specially favored in the presence of the catalysts, with yields 58% higher than those achieved in control reactions. This provided strong evidence that CHX is metabolized to pCA upon ingestion.

1. Introduction

Chlorhexidine (CHX, Fig. 1) is a bis-guanidine with bactericidal and fungicidal properties. It is commonly used in surgical [1–3], neonatal treatments, periodontal treatments [4,5], and oral rinses [6], and it is also employed as additive in chicken and pig food, among other applications. Although the skin absorption of CHX is not significant, as documented in the literature [7], the use of this compound as preservative in chicken meat or food as well as in rinse solutions might lead to the generation of toxic metabolites [8] such as *p*-chloroaniline (pCA) and *p*-chloronitrobenzene (pCNB) [8–12] when such food is consumed.

The oxidative metabolism of exogenous compounds in plants, animals, bacteria, and fungi is mediated by a super family of cytochrome P450 enzymes [13], which have an iron protoporphyrin IX as the prosthetic group (Fig. 2).

The iron(IV)-oxo porphyrin π-cation, a highly eletrophilic species, is assumed to be the most important catalytic intermediate in reactions catalyzed by cytochrome P450 enzymes. However, the general consensus nowadays is that this species may not be the only catalytic intermediate responsible for the large number of reactions mediated by the cytochromes P450, as reported in recent works [14,15]. The existence of different catalytic species enables the cytochromes P450 to carry out a wide variety of chemical transformations, including countless reactions like alkene epoxidation, *n*-dealkylation of secondary and tertiary amines, *o*-dealkylation, and hydroxylation of aromatic compounds, among others [13,16].

A number of biomimetic systems that are able to mimic the function of P450 enzymes have been developed, in order to contribute to a better understanding of the action mechanisms of these enzymes [13,17]. Synthetic metalloporphyrins have been successfully used as P450 models for the oxidation of many endogenous and exogenous compounds, mainly for comparison purposes and identification of the metabolites formed in *in vivo* systems and/or as an alternative method for the production of these metabolites.

The toxicity of the organochloride metabolites of chlorexidine [18–20] justifies studies involving CHX degradation or CHX metabolization by cytochrome P450 in living organisms. However, the great complexity inherent to the study of *in vivo* systems, the way metalloporphyrins successfully mimic the cytochrome P450 enzymes, and our ongoing interest in this field have prompted the present investigation on the use of metalloporphyrins as cytochrome P450 models for the prediction and identification of the possible metabolites generated from the antimicrobial agent CHX.

2. Experimental

2.1. Physical measurements

UV–vis spectra were obtained on a Hewlett–Packard 8452A diode array spectrometer. Analytical HPLC analyses were

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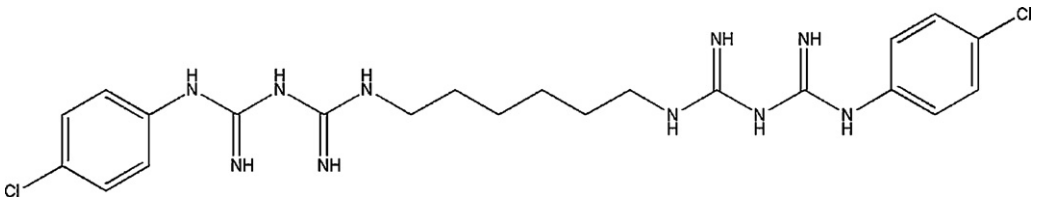

Fig. 1. Chlorhexidine, CHX.

performed on a SHIMADZU liquid chromatograph equipped with an LC-10AS solvent pump, an SPD-M 10A VP spectrophotometric detector (λ = 216 nm for pCA and pCNB, and 234 nm for CHX) coupled to a CTO-10A VP column oven, and an SCL-10A VP system controller. Separation of CHX and the oxidation products pCA and pCNB was carried out in a C18 Shim-pack CLC-ODS (M) column with a particle size of 5 μm (250 mm × 4 mm) supplied by Merck, using a trifluoroacetic acid 0.08% acetonitrile/aqueous solution (v/v) as eluent. The analytical column was protected by a Lichrospher guard column (4 mm × 4 mm). GC–MS was conducted on a QP2010 mass spectrometer (Shimadzu) fitted with a GC17A gas chromatograph (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was accomplished in the temperature-programming mode, using a DB-5MS column (30 m × 0.25 mm × 0.25 μm). Reaction products were identified by comparison of their retention times with known reference compounds, and by comparing their mass spectra to fragmentation patterns obtained from the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the mass spectrometer.

2.2. Materials

Unless otherwise stated, all the compounds used herein were purchased from Aldrich or Merck and were of analytical grade. The porphyrins 5,10,15,20-*tetrakis*(4-carboxyphenyl)porphyrin, H_2(TCPP), and 5, 10, 15, 20 *tetrakis*(4-N-methylpyridil)porphyrin, H_2(TMPyP), were acquired from Mid-Century. Iron insertion into these free base-porphyrins was carried out by using the method of Adler et al. [21]. *tert*-Butyl hydroperoxide (70 wt.% solution in water) and 3-chloroperoxybenzoic acid were provided by Acros Oganics. Hydrogen peroxide (H_2O_2, 30% in water) was supplied by Fluka and stored at 5 °C, and it was periodically titrated for confirmation of its purity. Acetonitrile (ACN) HPLC grade was obtained from Mallinckrodt. Water used in the experiments was purified by a Milli-Q, Millipore System.

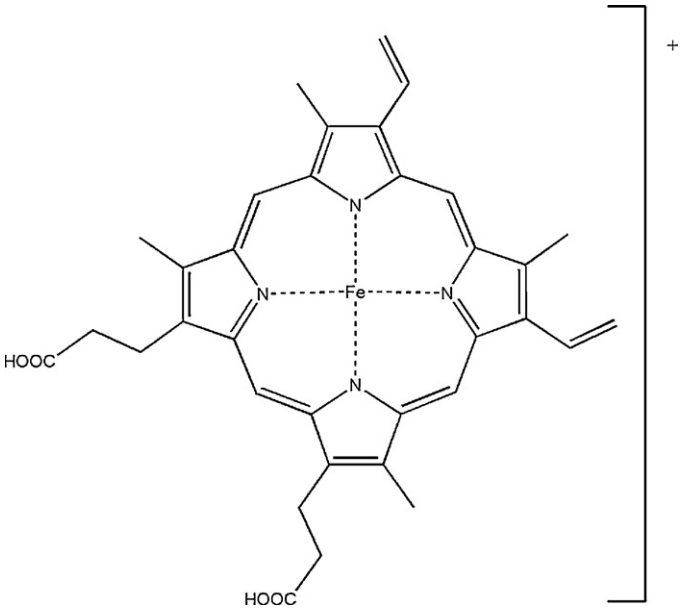

Fig. 2. Iron-protoporphyrin IX.

2.3. Oxidation reactions

Reactions were carried out in a 3-mL vial containing a screw cap. Briefly, CHX (56 μL, 1.25×10^{-5} mol), 2.5×10^{-7} mol of the metalloporphyrin solubilized in 50 μL water, and the oxidant (*m*CPBA, *t*BuOOH, or H_2O_2, 5.0×10^{-5} mol) in 2 mL water were added to a reaction vial. Reactions were carried out for 24 h, under magnetic stirring at room temperature, at a catalyst/oxidant/CHX molar ratio of 1:200:50, which was the initial condition in our studies. Other catalyst/oxidant/CHX molar ratios were also employed. At the end of the reaction, magnetic stirring was interrupted, and an aliquot of the reaction mixture (50 μL) was withdrawn and analyzed by high-performance liquid chromatography (HPLC) or GC–mass. The pH effect was investigated in buffered aqueous solution. Reactions at pH 3 and 10 were performed in acetic acid 0.1 mol L^{-1}, and in carbonate buffer, respectively. The pH of the reaction solution was adjusted by adding either HCl (0.5 mol L^{-1}) or NaOH (0.5 mol L^{-1}) solutions whenever necessary.

The oxidation products were identified by comparison of their retention times with those of authentic standards. Yields (or conversion) are based on the added drug and were determined by means of a calibration curve. Other minor products were identified by mass spectrometry, although their structural elucidation is still not conclusive.

Control reactions were carried out in the absence of the catalyst, under the same conditions as the catalytic runs.

3. Results and discussion

The metalloporphyrins FeTMPyP and FeTCPP (Fig. 3a and b, respectively) were chosen for these studies because they are commercially available and display good catalytic activity, as well described in the literature [23,24]. Moreover, FeTMPyP and FeTCPP exhibit appreciable water solubility, which enables their use in studies carried out in aqueous medium. Hydrogen peroxide was the oxidant of choice since it is clean, yields only water as byproduct, and provides useful information for mechanism proposition.

The initial reactions for the investigation of CHX oxidation by H_2O_2 were carried out using 2.5×10^{-7} mol catalyst at a catalyst/oxidant/CHX ratio of 1:200:50, which had been previously determined as standard conditions for other catalytic systems [22], in aqueous medium. Reaction products were analyzed by HPLC. In these conditions, one compound was detected as the main degradation product, and it displayed the same elution time and UV/Vis spectrum as an authentic *p*-chloroaniline (pCA) sample. Some other minor products were also verified.

To confirm that pCA was the main product and in an attempt to identify the minor products, the reaction products were also analyzed by GC–MS. An authentic sample of pCA was also injected

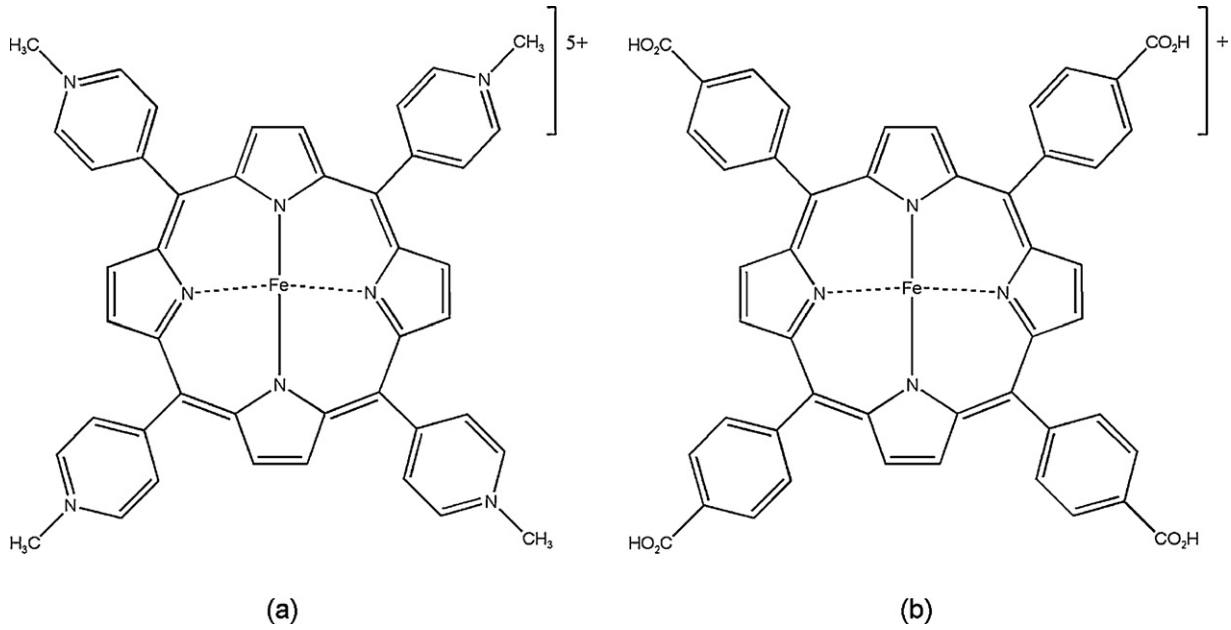

Fig. 3. (a) 5,10,15,20-*tetrakis*(4-N-methylpyridil)porphyrin iron (III), FeTMPyP; (b) 5,10,15,20 *tetrakis*(4-carboxyphenyl)porphyrin iron (III), FeTCPP.

under the same conditions, for comparison of the elution times and fragmentation patterns in relation to the main reaction product.

There was a perfect correlation between the retention times and the fragmentation patterns obtained for the main reaction product and the authentic pCA sample (retention times of 8.00 and 7.98 min respectively and mass fragments of 65, 92 and 127/129 u for both samples), thus confirming that the main CHX degradation product in these conditions was pCA.

Once pCA formation was ratified, this product was quantified by HPLC under different reaction conditions, by means of a calibration curve constructed from authentic pCA samples.

Table 1 summarizes the pCA yields under different conditions. pCA formation was also detected in control reactions conducted in the absence of the catalyst. Hence, the last column in Table 1 as well as in the other tables included in this work refers to the pCA formed exclusively through catalyst action and was calculated *via* the following Eq. (1):

$$\text{pCA yield}\,(\%) = \left(\frac{[\text{pCA}]_{\text{cat}} - [\text{pCA}]_{\text{ncat}}}{[\text{pCA}]_{\text{ncat}}}\right) \times 100\%, \quad (1)$$

where $[\text{pCA}]_{\text{cat}}$ = pCA concentration in the catalyzed reaction and $[\text{pCA}]_{\text{ncat}}$ = pCA concentration in the non-catalyzed reaction.

Increasing oxidant concentration implied in higher pCA yields up to a catalyst/oxidant/substrate molar ratio of 1:2500:50 (Table 1). The pCA yields decreased thereafter, when there was fast discoloration of the reaction medium, which indicated catalyst degradation. This was confirmed through monitoring of the reaction by UV/Vis spectroscopy at regular time intervals. After 4 h of reaction, the Soret band at 426 nm, characteristic of the catalyst, disappeared, thus corroborating destruction of the metalloporphyrin.

Table 1
pCA yields obtained from the CHX oxidation by H_2O_2 catalyzed by FeTCPP in aqueous medium.

Catalyst/oxidant/substrate molar ratio[a]	Total pCA yield ($[\text{pCA}]_{\text{cat}}$)[b] (%)	pCA yields[c] (%)
1:200:50	15	49
1:300:50	18	49
1:2500:50	60	58
1:5000:50	65	41
1:10,000:50	92	10

[a] Catalyst = 2.5×10^{-7} mol, yields after 24 h of reaction.
[b] Total pCA yields obtained in reactions.
[c] pCA yields as determined by Eq. (1).

These results attested to the fact that, although pCA formation occurred even in the absence of the catalyst, this formation was considerably higher when the reaction was catalyzed by metalloporphyrins. In conditions where there was a balance between oxidant concentration and catalyst lifetime (*i.e.*, 1:2500:50), the difference between the catalyzed and the non-catalyzed reaction was more significant, as well as the total pCA yield. Considering these results, the 1:2500:50 molar ratio was used for evaluation of the effects of other variables on the CHX oxidation, unless otherwise stated.

Catalytic pCA formation may take place by different mechanisms, depending on the generated active intermediate species. As reported in the literature, two catalytic species can generally be formed as a result of the interaction of the metalloporphyrin with hydrogen peroxide, as shown in Fig. 4 [14,23,24]. The main one is the iron$^{(IV)}$-oxo-porphyrin π-cation radical, $Fe^{(IV)}OP^{\bullet+}$ (II, Fig. 4), which acts as a strong electrophile and performs oxidations of various functional groups such as alcohols and alkenes, among others [13,25,26]. The other possible catalytic species is the iron$^{(III)}$-hydroperoxo porphyrin, $Fe^{(III)}$–OOH(P) (I, Fig. 4), a nucleophilic species that can be formed when the proton donation that leads to $Fe^{(IV)}OP^{\bullet+}$ is inhibited [14,23,25]. Considering these species, one possible mechanism accounting for pCA formation in the studied systems could involve the oxidation of the CHX iminic bond by the highly electrophilic intermediate $Fe^{(IV)}OP^{\bullet+}$, yielding a oxaziridine ring (**1a**, Fig. 4), which hydrolyzes and furnishes pCA and a N-(hydroxy)amide (**1b**, Fig. 4).

Another possibility involves the nucleophilic intermediate iron-hydroperoxo porphyrin (**I**, Fig. 4), which preferably attacks the CHX iminic carbon, thereby affording intermediate **2a**, Fig. 4. An intramolecular rearrangement of intermediate **2a** furnishes pCA and an amide (**2b**, Fig. 4).

As neither amide **2b** (Fig. 4) nor the N-(hydroxy)amide **1b** could be isolated, there was no conclusive evidence of which mechanism is responsible for pCA formation in these systems. However, some indication about which mechanism predominates in CHX

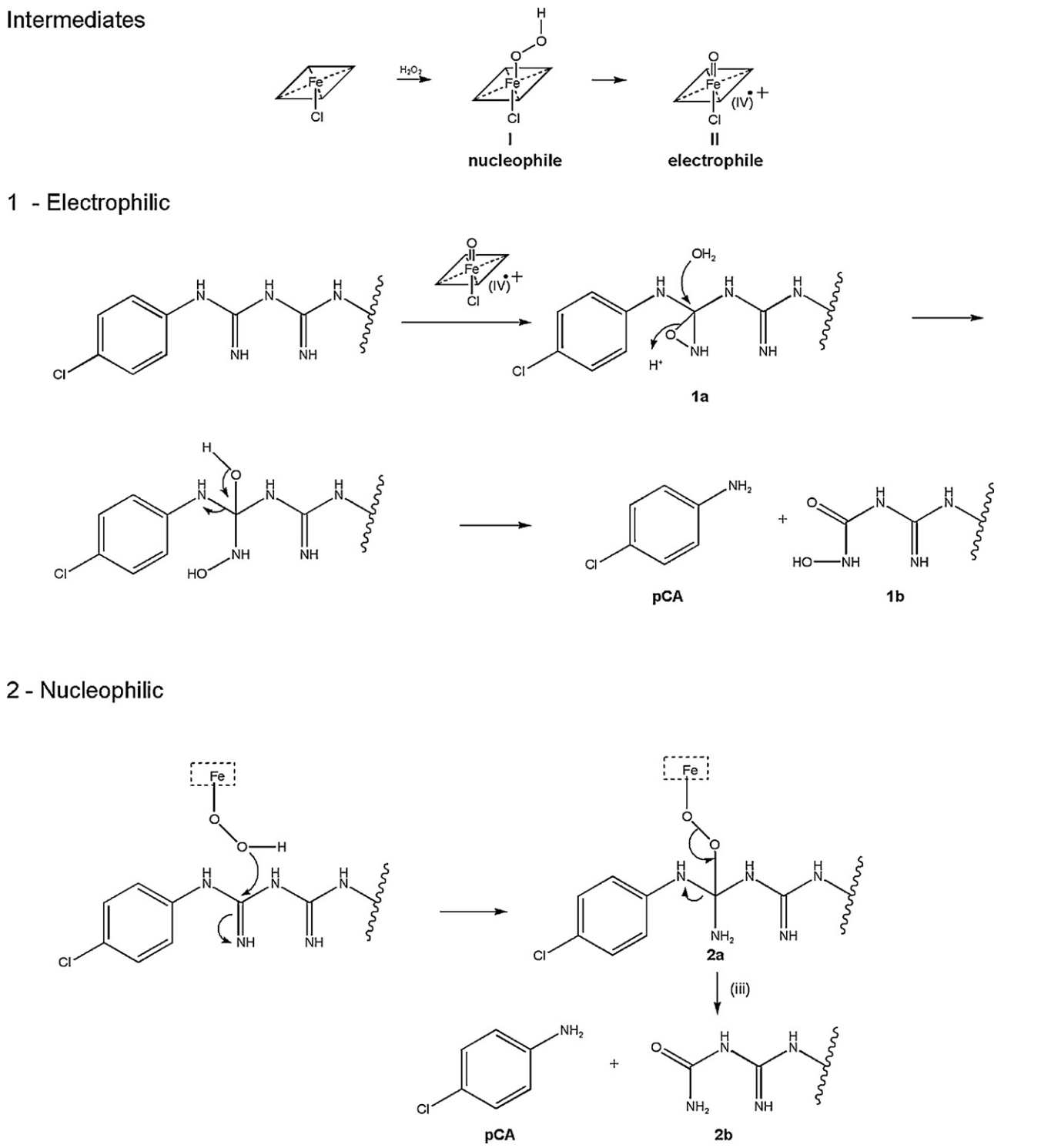

Fig. 4. Possible mechanisms of pCA formation from CHX degradation by H_2O_2 mediated by metalloporphyrins, in aqueous medium: (1) electrophilic pathway involving $Fe^{(IV)}OP^{\bullet+}$ and (2) nucleophilic pathway through the $Fe^{(IV)}OOH(P)$ species.

degradation can be obtained from the studies of reaction variables such as pH and oxidant nature.

Table 2 presents the pCA yields attained from CHX oxidation reactions mediated by FeTCPP under different pH values.

The pH had little effect on pCA yields for the non-catalyzed reactions (36% pCA in acid pH *vs* 38% in neutral pH), but it played a significant role in reactions occurring in the presence of the metalloporphyrins. This corroborated the fact that these complexes actively participated in pCA formation, and that this participation was also pH-dependent. Results listed in Table 2 revealed a reduction in or absence of pCA at both extreme pH values for reactions accomplished in the presence of the catalyst.

An alkaline pH probably reduces the availability of CHX in the reaction medium, because of the minor solubility of this substrate in

Table 2
pCA yields from CHX oxidation by H_2O_2 mediated by FeTCPP under different pH values, in aqueous medium.

pH	Total pCA yield (%)		pCA yield[b] (%)
	Non-catalyzed	Catalyzed[a]	
3	36	41	13
7	38	60	58
10	2	2	0

Catalyst/oxidant/substrate 1:2500:50. Catalyst = 2.5×10^{-7} mol, yields after 24 h of reaction.
[a] pCA yields obtained in catalyzed reactions.
[b] pCA yields as determined by Eq. (1).

these conditions. As a consequence, the pCA yields are considerably diminished in this case (Table 2).

Reduced pCA formation under acid medium is coherent with the nucleophilic mechanism (**2**, Fig. 4), since higher proton concentration favors protonation of the nucleophilic iron-hydroperoxo species (**I**, Fig. 4), leading to fast consumption of this intermediate and consequent $Fe^{(IV)}OP^{\bullet+}$ production, thereby reducing the probability of a nucleophilic attack on the CHX molecules.

In neutral medium, the nucleophilic mechanism is also favored as a result of the higher lifetime of the hydroperoxo species, since the cleavage of the O—O bond that culminates in the iron$^{(IV)}$-oxo-porphyrin π-cation radical is considerably slower. In conclusion, the pH effects suggest that the nucleophilic attack prevails, since the catalytic yields rose substantially under neutral medium, where the iron-hydroperoxo species has longer half-life.

The utilization of different oxidants is also interesting because each one favors a different catalytic intermediate, thus providing some useful information about the reaction mechanisms. In this context, H_2O_2 was substituted by *m*-chloroperbenzoic acid (*m*CPBA), *tert*-butyl-hydroperoxyde (*t*BuOOH), or sodium hypochlorite (NaOCl) in equimolar amounts, and FeTMPyP (Fig. 3a) was employed as the catalyst, for evaluation of their effects on CHX oxidation.

Table 3 shows that although the H_2O_2 was not the best oxidant in terms of total pCA yields, it was the most efficient in the case of the catalyzed reactions. It also accounts for the nucleophilic mechanism, because this oxidant is the only reagent that is able to generate iron-hydroperoxo, the active species in the nucleophilic mechanism (Fig. 4).

The high pCA yields observed for *m*CPBA in the non-catalyzed reactions can be explained by the direct epoxidation of the iminic double bond, to yield an oxaziridine [27]. Epoxide ring-opening induced by a nucleophilic attack of a water molecule would yield a N-(hydroxy)amide and pCA similarly as shown in Fig. 4, **I** where the electrophilic mechanism in the presence of the metalloporphyrins

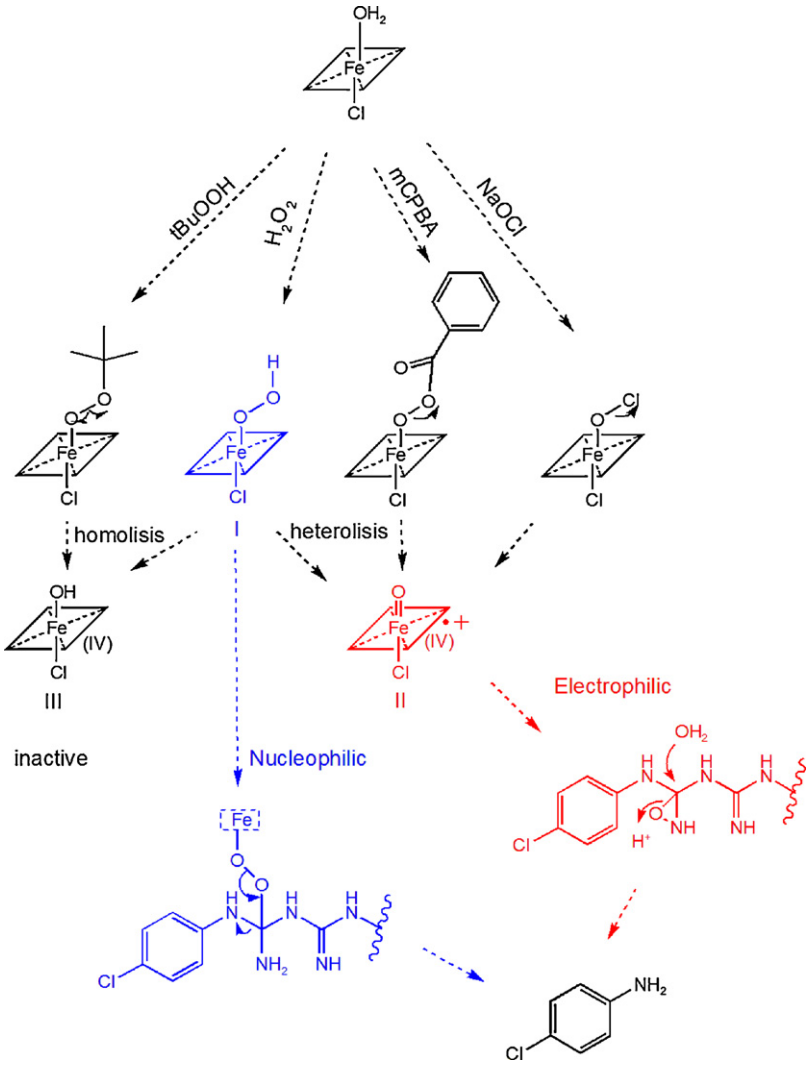

Fig. 5. Main catalytic species probably involved in the CHX oxidation reactions by different oxidants catalyzed by ironporphyrins.

Table 3
pCA yields from CHX oxidation by different oxidants mediated by FeTMPyP in aqueous medium.

Oxidant	Total pCA yield[a] (%)	pCA yields[b] (%)
H_2O_2	12	71
*m*CPBA	24	9
NaOCl	21	−23
*t*BuOOH	4	33

Catalyst/oxidant/substrate 1:150:50, 24 h.
[a] pCA yields for catalyzed reactions.
[b] Calculated using Eq. (1).

is described. In this case, however, the electrophilic species is the *m*CPBA instead of the $Fe^{(IV)}OP^{\bullet+}$.

Although NaOCl led to high pCA yields, these results were lower compared to those achieved in the case of the non-catalyzed reactions in the same conditions (Table 3). pCA formation in this case may have taken place through CHX alkaline hydrolysis, as suggested in the literature [8,28,29]. Hydroxyl ions originated from OCl^- hydrolysis in aqueous medium may have attacked the CHX iminic carbon, thereby generating pCA. Part of the OCl^- ions was consumed in the presence of the metalloporphyrins, to form the iron$^{(IV)}$-oxo-porphyrin π-cation radical, as depicted in Fig. 5. This reaction probably competed with the fast CHX hydrolysis, thus resulting in lower pCA yield.

Lower pCA yields were also achieved in the CHX oxidation by *t*BuOOH, as compared to H_2O_2 (Table 3). These results can also be explained by analysis of the main intermediate yielded by this oxidant. Alkylhydroperoxides often tend to undergo homolytic O—O bond cleavage upon coordination to the metalloporphyrin [30,31], thereby giving rise to a radical (*t*BuO·) and the $Fe^{(IV)}$POH species (III, Fig. 5), which is not able to epoxidize or perform a nucleophilic attack on CHX [25].

Fig. 5 contains a scheme showing the main catalytic species that is probably responsible for CHX oxidation by the different oxidants for the reactions carried out in the presence of the ironporphyrins.

In the case that the electrophilic mechanism acted during CHX oxidation, the results obtained for NaOCl and *m*CPBA suggested that both oxidants were strong enough to directly oxidize the iminic double bond, as compared to the iron$^{(IV)}$-oxo-porphyrin π-cation radical. For H_2O_2 and *t*BuOOH, however, participation of the catalysts was crucial to pCA formation, since these oxidants alone are unable to epoxidize the iminic double bond. Therefore, the ability to form the iron$^{(IV)}$-oxo-porphyrin π-cation radical intermediate was most important in this case. When it is coordinated to the metalloporphyrin, hydrogen peroxide is easily protonated, hence favoring the O—O bond cleavage and furnishing higher pCA yields, as compared to *t*BuOOH.

Although pH and oxidant effects indicated the predominance of a nucleophilic mechanism during CHX oxidation by H_2O_2, other studies are necessary to prove these mechanisms. For example, spectroscopic investigation of the reaction intermediates is under way in our laboratory.

4. Conclusion

The antiseptic agent chlorhexidine is prone to oxidative degradation by hydrogen peroxide, *m*CPBA, *t*BuOOH, and NaOCl, yielding pCA as the main product. In the presence of metalloporphyrins, pCA formation rises substantially as compared to the non-catalyzed reactions. The higher pCA yields amount to 71% in the following conditions: catalyst/oxidant/substrate molar ratio of 1:150:50, aqueous medium, FeTMPyP as catalyst.

It is also remarkable that under physiological pH, pCA formation is specially favored in the presence of the metalloporphyrins (with yields 58% higher than that in control reactions), thus furnishing strong evidence that this product may be a CHX metabolite when the antiseptic is ingested.

Based on the pCA yields under different reaction conditions, it is difficult to unambiguously determine which of the mechanisms is responsible for pCA formation, one involving the iron$^{(IV)}$-oxo-porphyrin π-cation radical intermediate or another mediated by a nucleophilic attack of the iron$^{(III)}$-hydroperoxo-porphyrin. However, an alert remains: under all the studied conditions pCA was detected as the main product. Considering the success of the metalloporphyrins as a synthetic analog of the cytochromes P450, the higher catalytic activity of these complexes in physiological pH demonstrates the strong possibility of pCA formation when CHX is ingested. Since pCA is highly toxic, application of CHX as preservative in chicken meat or as food additive may require revised regulation and reinforces the need for more detailed studies on CHX toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apcata.2012.08.026.

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