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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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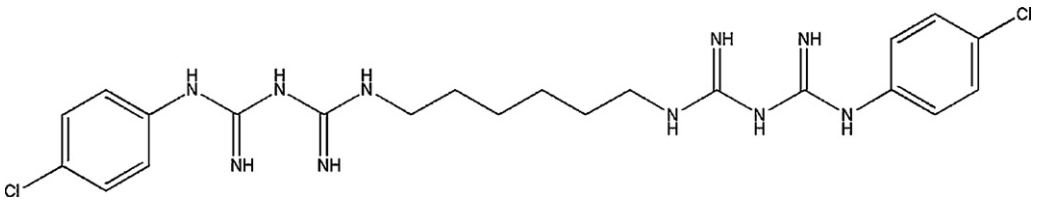

Fig. 1. Chlorhexidine, CHX.

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.

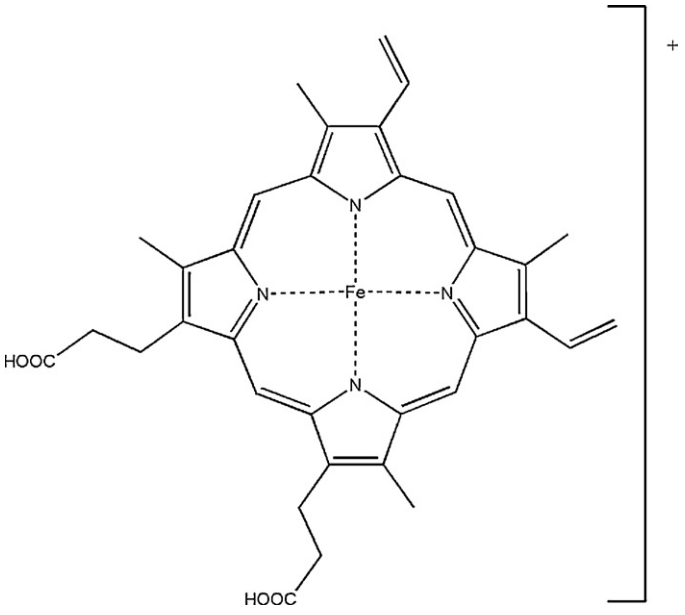

Fig. 2. Iron-protoporphyrin IX.

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