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Oxidation of 4-Chloroaniline Catalyzed by Human Myeloperoxidase

A. R. J. BAKKENIST, H. PLAT, AND R. WEVER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands

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The incubation of 4-chloroaniline with H_2O_2 and myeloperoxidase results in the formation of at least 10 products. Possibly some structures with high complexity, like 4,4'-dichloroazobenzene, are present; however, no 4-chloronitrosobenzene is detectable. This result contrasts with the oxidation of 4-chloroaniline catalyzed by chloroperoxidase, which only yields 4-chloronitrosobenzene.

INTRODUCTION

Myeloperoxidase, the heme-containing peroxidase of polymorphonuclear leukocytes, catalyzes in the presence of H_2O_2 the oxidation of classical peroxidase electron donors such as *o*-dianisidine and guaiacol (*1*). Moreover, when both H_2O_2 and chloride ion are present, the enzyme also has the ability to form hypochlorous acid, which chlorinates a number of compounds (*2, 3*). Most other peroxidases such as horseradish peroxidase do not catalyze this chlorinating reaction. There is another enzyme known that can chlorinate a broad range of nucleophilic-acceptor substrates, i.e., chloroperoxidase (EC 1.11.1.10) (*4*). This enzyme, which is found in soil fungi and aquatic marine organisms, has another unique property, namely its ability to convert arylamines into aromatic nitroso compounds (*5–7*). In particular, the enzyme-catalyzed peroxidation of 4-chloroaniline has been studied (*6, 7*). Chlorinated anilines are common environmental residues resulting from the degration of pesticides and herbicides. Other peroxidases which are present in soil and plants can convert these residues to azobenzenes and to polyaromatic products of higher complexity (*8*).

It is known that intravenous administration of 4-chloroaniline to dogs results in the formation of significant amounts of the metabolite 4-chloronitrosobenzene (*9*). However, the route of formation of this probably carcinogenic product is unknown.

Since myeloperoxidase, like chloroperoxidase, is able to oxidize chloride ion to hyochlorous acid, it is conceivable that after uptake of chlorinated anilines in the body myeloperoxidase is involved in the oxidation of these compounds. In this paper we report the myeloperoxidas-catalyzed reaction between 4-chloroaniline and H_2O_2 and the nature of the products formed.

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MATERIALS AND METHODS

Myeloperoxidase was isolated from human granulocytes as described before (*10*). The enzyme concentration was calculated with an absorbance coefficient of 89 $mM^{-1} \cdot cm^{-1}$ at 428 nm. The absorbance ratio ($A_{428\,nm}/A_{280\,nm}$) of the enzyme was 0.9.

Chloroperoxidase was obtained from Sigma Chemical Company, 4-chloroaniline from British Drug Houses, and ammonium disodium pentacyanoamine ferroate from Flucka. Hydrogen peroxide solutions were freshly prepared by dilution of a 30% stock solution (Merck). The stock solution was periodically standardized by permanganate. Silica gel-precoated thin-layer plates (Polygram Sil G/UV254) were obtained from Macherey Nagel and Company, Düren, Federal Republic of Germany. All other chemicals were of analytical grade. Spectrophotometric measurements were carried out on a Cary-17 spectrophotometer.

The oxidation of 4-chloroaniline was started by the addition of myeloperoxidase to a cuvette-containing buffer, H_2O_2 and 4-chloroaniline.

To identify the oxidation products, 2 m*M* 4-chloroaniline was incubated with 50 μM H_2O_2 and 66 n*M* myeloperoxidase in 0.1 *M* potassium phosphate buffer (pH 6.5) during 4 hr at room temperature. The incubation of chloroperoxidase was carried out as described by Corbett *et al.* (*5*). Water in the incubation mixtures was removed by evaporation under reduced pressure. The residues were taken up in methanol from which portions were chromatographed on silica gel thin-layer plates in three solvent systems: hexane, chloroform, and hexane/toluene (2: 1, v/v). The noncolored products were detected by quenching irradiation at 254 and 360 nm, and by spraying with ammonium disodium pentacyanoamine ferroate, a reagent specific for 4-chloronitrosobenzene (*5*).

RESULTS

The addition of chloroperoxidase to a solution containing 1.2 m*M* 4-chloroaniline and 2 m*M* H_2O_2 at pH 4.5 resulted in the formation of a product with one stable absorbance maximum at 320 nm (Fig. 1). This product was identified by Corbett *et al.* (*5, 6*) as 4-chloronitrosobenzene. However, when myeloperoxidase was added to a mixture of 4-chloroaniline and H_2O_2 at pH 6.5, products were formed with optical absorbance maxima at 312 and 420 nm. These maxima shifted in 30 min to longer wavelengths, 320 and 450 nm, respectively, and became more intense (Fig. 1). The color of the solution changed from yellowish-red to red, and a red-brown solid oxidation product precipitated. The same phenomena were observed when chloride ion was present in the incubation mixture, or when the pH was changed to pH 4.5.

The rate of enzymic oxidation of 4-chloroaniline by H_2O_2, followed as an absorbance change at 312 nm, increased linearly with the amount of myeloperoxidase added. When enzyme or H_2O_2 was omitted, no reaction occured. Figure 2A shows that the oxidation reaction was extremely sensitive to H_2O_2, both K_m and K_i are low. A Michaelis constant of 3.4 μM for H_2O_2 at 2.0 m*M* 4-chloroaniline

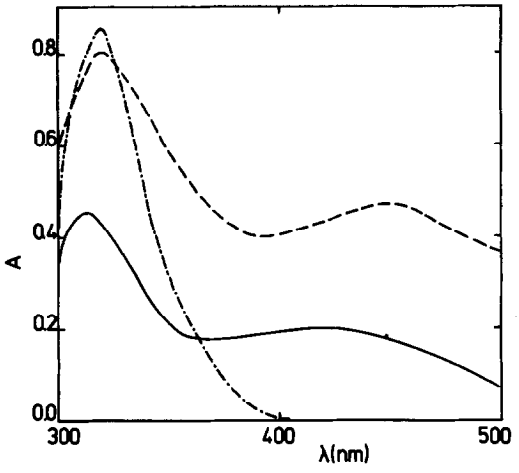

FIG. 1. Absorbance spectra of the 4-chloroaniline oxidation products. 4-Chloroaniline, 2.0 m*M*, was incubated with 50 μM H_2O_2 and 66 n*M* myeloperoxidase in 0.1 *M* potassium phosphate (pH 6.5). The spectra were recorded immediately (———) and 30 min (– – –) after addition of myeloperoxidase. (– · – · –) Incubation of 1.2 m*M* 4-chloroaniline with 2.0 m*M* H_2O_2 and 10 n*M* chloroperoxidase in 0.1 *M* sodium acetate (pH 4.5).

and pH 6.5 can be calculated from a Lineweaver–Burk plot using the uninhibited part of the curve in Fig. 2A.

The inhibition of the reaction by higher concentrations of H_2O_2 is a well-known event in peroxidase-catalyzed reactions (*2, 8*). The myeloperoxidase-catalyzed oxidation of 4-chloroaniline was inhibited, however, at 25 μM H_2O_2, whereas the same reaction catalyzed by chloroperoxidase was hardly affected at a H_2O_2 concentration of 5 m*M* (*6*). When chloride ion was present in the incubation mixture with myeloperoxidase, the initial reaction rate was accelerated. At 0.1 *M*

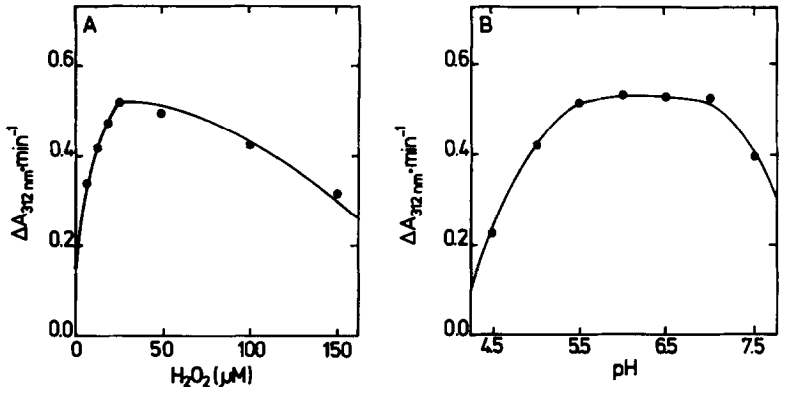

FIG. 2. (A) Rate of oxidation of 4-chloroaniline catalyzed by myeloperoxidase as a function of the H_2O_2 concentration. The reaction mixture contained 1.25 m*M* 4-chloroaniline, 0.1 *M* potassium phosphate (pH 6.5), 30 n*M* myeloperoxidase, and H_2O_2 as indicated in the figure. (B) pH dependence of the oxidation rate of 4-chloroaniline. 4-Chloroaniline, 1.25 m*M*, was incubated with 25 μM H_2O_2, 40 n*M* myeloperoxidase, and 0.1 *M* sodium acetate or potassium phosphate.

NaCl the apparent K_m for H_2O_2 was 2.8 μM, nearly the same as in the absence of chloride, but the V value was twice as high. Furthermore, the inhibition by H_2O_2 did not occur up to 50 μM H_2O_2. Thus chloride ion appears to protect the enzyme against the inactivation by H_2O_2, as was already suggested in studies of the chlorinating activity of myeloperoxidase (*2, 3*). Variation of the concentration of 4-chloroaniline revealed normal Michaelis–Menten kinetics. The apparent K_m for 4-chloroaniline at 25 μM H_2O_2 and pH 6.5 was calculated to be 10 mM.

Figure 2B shows that myeloperoxidase is able to catalyze optimally the oxidation of 4-chloroaniline over a broad pH range from pH 5 to pH7. Below pH 5 and above pH 7 the reaction rate slowed down, and at pH 3 no significant absorbance increase between 300 and 450 nm could be detected.

The aspects of the oxidation of 4-chloroaniline described were similar for the oxidation of aniline. Under identical reaction conditions the apparent K_m for H_2O_2 was 5.2 μM and for aniline 8.3 mM (not shown). Also the pH range for optimal aniline oxidation showed the same broad profile as for 4-chloroaniline oxidation.

The products formed in the oxidation of 4-chloroaniline by H_2O_2 catalyzed by myeloperoxidase or chloroperoxidase were chromatographed on thin-layer plates. When chloroform was used to develop the chromatogram, more than 10 spots could be detected in the solution of products formed by myeloperoxidase (Fig. 3). In contrast, only one spot was seen on the thin-layer plates in the incubation mixture with chloroperoxidase. Moreover, this product was not detectable on the myeloperoxidase chromatogram.

DISCUSSION

The oxidation of 4-chloroaniline catalyzed by myeloperoxidase results in the formation of many colored and noncolored products. Although identification of these products is very difficult, it seems likely that some of these compounds are identical to those which are formed in the peroxidation of 4-chloroaniline by horseradish peroxidase (*11*) and soil peroxidases (*8*). The absorbance spectrum of the product suggests the presence of 4,4′-dichloroazobenzene and 4-chloro-4′-(4-chloroanilino)azobenzene (*8*). If these compounds are indeed present, the solution should contain other products too, since on thin-layer chromatograms 10 components are detectable. However, even after spraying with ammonium disodium pentacyanoamine ferroate it was impossible to detect any trace of 4-chloronitrosobenzene, which is the only product of the same reaction catalyzed by chloroperoxidase. Although the oxidation of 4-chloroaniline by chloroperoxidase (*5–7*) and the oxidation of 4-chloroaniline by myeloperoxidase are both accelerated by chloride ion, it is clear that the mechanism of the reaction is different for both enzymes and that myeloperoxidase, like the classical peroxidases, is unable to catalyze a two-electron oxidation of these arylamines.

Whether the oxidation of chlorinated aniline and the formation of all products occur under physiological conditions, is not known. The pH and the myeloperoxidase concentration used in the experiments approach those present in serum (*12*). The results of the kinetic experiments (Fig. 2A) show that a low concentration of

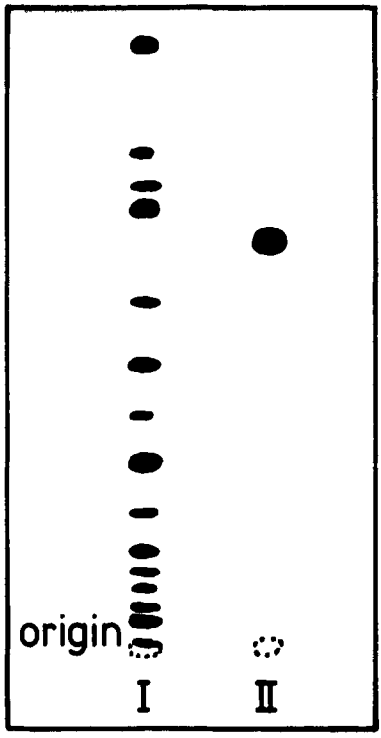

FIG. 3. Thin-layer chromatogram of the oxidation products of 4-choroaniline. Incubation conditions were the same as in Fig. 1. The chromatogram was developed as described under Methods. (I) Myeloperoxidase; (II) chloroperoxidase.

H_2O_2 is a prerequisite for enzymic oxidation of 4-chloroaniline. Since such low levels of H_2O_2 may exist *in vivo* it is likely that, when chloroamines are taken up or administered, some of these potentially harmful products are formed.

REFERENCES

1. S. J. KLEBANOFF AND R. A. CLARK, "The Neutrophil: Function and Clinical Disorders," pp. 409–488. North-Holland, Amsterdam, 1978.
2. A. R. J. BAKKENIST, J. E. G. DE BOER, H. PLAT, AND R. WEVER, *Biochim. Biophys. Acta* **613**, 337 (1980).
3. J. W. NASKALSKI, *Biochim. Biophys. Acta* **485**, 291 (1977).
4. J. A. THOMAS, D. R. MORRIS, AND L. P. HAGER, *J. Biol. Chem.* **245**, 3129 (1970).
5. M. D. CORBETT, B. R. CHIPKO, AND D. G. BADEN, *Biochem. J.* **175**, 353 (1978).
6. M. D. CORBETT, D. G. BADEN, AND B. R. CHIPKO, *Bioorg. Chem.* **8**, 91 (1979).
7. M. D. CORBETT, B. R. CHIPKO, AND A. O. BATCHELOR, *Biochem. J.* **187**, 893 (1980).
8. L. M. BORDELEAU AND R. BARTHA, *Canad. J. Microbiol.* **18**, 1873 (1972).
9. M. KIESE, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **245**, 484 (1963).
10. A. R. J. BAKKENIST, R. WEVER, T. VULSMA, H. PLAT, AND B. F. VAN GELDER, *Biochim. Biophys. Acta* **524**, 45 (1978).
11. B. C. SAUNDERS, A. G. HOLMES-SIEDLE, AND B. P. STARK, "Peroxidase," pp. 10–25. Butterworths, London, 1964.
12. T. OLOFSSON, I. OLSSON, AND P. VENGE, *Scand. J. Haematol.* **18**, 113 (1977).