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HYDROGEN CYANIDE AND CYANOGEN CHLORIDE FORMATION BY THE MYELOPEROXIDASE-H_2O_2-Cl^- SYSTEM

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Summary

The chlorination of glycine by the myeloperoxidase-H_2O_2-Cl^- system at acidic pH values yielded *N*-monochloroglycine and a mixture of HCN and ClCN. HCN was formed as a product of *N*-dichloroglycine decomposition and cyanogen chloride formation resulted from simultaneous chlorination of HCN by *N*-chloroglycine or directly by the myeloperoxidase-H_2O_2-Cl^- system.

HCN was readily chlorinated by the myeloperoxidase-H_2O_2-Cl^- system yielding cyanogen chloride. The dissociation constants of the myeloperoxidase-CN^- complex were estimated as $2.5 \cdot 10^{-6}$—$1.15 \cdot 10^{-5}$ M within the pH range 6.2 to 3.4, respectively. Chloride competed with cyanide for binding at the active site of myeloperoxidase. The lower the pH the more pronounced was the competitive effect of chloride. This accounted for chlorination by myeloperoxidase in the presence of CN^-.

Introduction

Myeloperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) catalyses the oxidation of Cl^- to HOCl by H_2O_2 [1—3]. This implies that the myeloperoxidase-H_2O_2-Cl^- system has chlorinating properties [2,4]. It may be assumed that the oxidation of Cl^- and further chlorination of biological important compounds is of crucial importance for the bactericidal properties of the myeloperoxidase-chloride-dependent enzymatic system of neutrophilic granulocytes [4,5].

In this paper the *N*-chlorination of glycine is presented. This process resulted in several secondary reactions of *N*-chloramines of glycine, which yielded CN^- and cyanogen chloride. HCN appeared to be a substrate chlorinated by the myeloperoxidase-H_2O_2-Cl^- system.

Materials and Methods

Myeloperoxidase was obtained from neutrophilic granulocytes [6]. The enzyme preparation has an absorption coefficient ratio $A_{430nm}/A_{280nm} = 0.6$ and a specific activity of 50 guaiacol units/mg [7]. For the determination of myeloperoxidase concentration, an absorption coefficient of 89 $mM^{-1} \cdot cm^{-1}$ at 430 nm was used [8].

Glycine was obtained from Hoffmann-la Roche (Basel, Switzerland), NaCN from Chemapol (Prague, Czechoslovakia). Hydrogen peroxide and NaCl were reagent grade products from P.O.Ch. (Gliwice, Poland). HOCl solutions of the required concentration and pH were prepared prior to use from a 0.15 M NaOCl stock solution and standardized iodometrically [9]. The other reagents were of analytical grade from P.O.Ch. Spectrophotometric measurements were carried out with UV-VIS Spectrophotometer SP-800 and scale expansion accessory SP-850 with additional recorder SP-200 (Pye Unicam, Cambridge, U.K.). The cuvette path lengths were 1 cm or 4 cm.

HCN concentration was estimated according to the method of Boxer and Rickards [10], modified as follows: 0.4 ml 0.017% chloramine T in 0.2 M phosphate buffer (pH 7.0) was added to 0.2 ml sample. After 2 min, 1.5 ml 1-phenyl-3-methylpyrazolone-5-pyridine reagent [10] were added. After 45 min, the absorbance at 630 nm was measured. The same procedure was used for cyanogen chloride substituting 0.2 M phosphate buffer instead of chloramine T solution.

Results and Discussion

Enzymatic chlorination of glycine was carried out at two different pH values, 4.8 and 5.7 (Table I).

TABLE I

THE PRODUCTS OF GLYCINE CHLORINATION BY THE MYELOPEROXIDASE-H_2O_2-Cl^- SYSTEM

The reaction mixture, 4 ml 50 mM phosphate/citrate buffer contained: 6 μmol glycine, 10 μg myeloperoxidase, 400 μmol NaCl; at pH 5.7, 7.2 μmol H_2O_2 added in 24 successive equal portions at 30—45 s intervals; at pH 4.8, 7 μmol H_2O_2 added in five successive equal portions at 2—3 min intervals.

pH	H_2O_2 (μmol)	$H_2C(NHCl)COOH$ (μmol)	HCN (μmol)	ClCN (μmol)
5.7 *	1.2	1.16	0	0
	2.4	2.2	0	0
	3.6	3.3	0	0
	4.8	4.0	0.05	0.1
	6.0	4.1	0	0.2
	7.2	4.2	0	0.25
4.8	1.4	0.7	0.08	0.12
	2.8	1.35	0.1	0.32
	4.2	1.95	0.05	0.63
	5.6	2.55	0.0	0.8
	7.2	2.8	0.0	1.07

* Concentration of products estimated after every fourth portion of H_2O_2.

The affinity of myeloperoxidase to Cl^- increases with a decrease in pH [4], but K_m values for H_2O_2 increase with an increase in H^+ and Cl^- concentrations [11]. Thus, the chlorination proceeds with optimal velocity, independent of pH, if the ratio of $[H_2O_2]$ to $[Cl^-]$ is maintained according to the empirical equation [11]:

$$\frac{[H_2O_2]}{[H^+] \cdot [Cl^-]} = 560\text{—}660\ M^{-1} \qquad (1)$$

Practically, at the different pH values in the presence of constant $[Cl^-]$ the required concentration of H_2O_2 for the same, optimal velocity of chlorination may be calculated from Eqn. 1.

At the pH values employed (Table I) in the presence of 0.1 M NaCl the optimal concentrations of H_2O_2 were low (0.13 mM at pH 5.7 and 1.05 mM at pH 4.8). If a substantial accumulation of the chlorinated product was necessary, hydrogen peroxide had to be added gradually in successive portions. In these conditions there was a stoichiometric formation of HOCl from H_2O_2 (Table I).

The enzymic chlorination of glycine resulted in conversion to *N*-monochloroglycine, which had, as described for other *N*-monochloroamino acid [2], an absorption maximum in the 250 nm region with $\epsilon_{254nm} = 370\ M^{-1} \cdot cm^{-1}$. At pH 5.7 the quantity of *N*-monochloroglycine corresponded to the amount of H_2O_2 added (Table I). At pH 4.8 a lower concentration of *N*-monochloroglycine resulted from the increased acid-catalysed dismutation of *N*-mono- to *N*-dichloramine [12]:

$$\begin{matrix} CH_2\text{-}NHCl \\ | \\ COOH \end{matrix} + H^+ \rightarrow \begin{matrix} CH_2\text{-}NH_3^+ \\ | \\ COOH \end{matrix} + \begin{matrix} CH_2\text{-}NCl_2 \\ | \\ COOH \end{matrix} \qquad (2)$$

Formation of HCN and cyanogen chloride

The *N*-dichloroamino acids are unstable compounds and decompose to the corresponding nitriles [13]. In the particular case of *N*-dichloroglycine HCN should be the product of degradation according to the equation:

$$\begin{matrix} CH_2\text{-}NCl_2 \\ | \\ COOH \end{matrix} \xrightarrow{-2\ HCl} \begin{matrix} C{\equiv}N \\ | \\ COOH \end{matrix} \rightarrow HCN + CO_2 \qquad (3)$$

In accordance with reaction 3 increasing quantities of HCN and ClCN were observed during the enzymatic chlorination of glycine, mainly at lower pH values (Table I). Cyanogen chloride formation was made possible by the chlorination of HCN by *N*-monochloroglycine or by the myeloperoxidase-H_2O_2-Cl^- system itself.

The detection of HCN and ClCN as the products of glycine chlorination confirmed the assumption that *N*-dichloroglycine was formed as an intermediate. To show that *N*-dichloroglycine is a parent compound for HCN and ClCN non-enzymatic synthesis of *N*-dichloroglycine was performed. Upon the addition of a two-fold molar excess of HOCl to glycine its dichloramine was obtained. It was characterized by an absorption in the region of 300 nm as reported for

dichloramines [4]. As expected it was an unstable compound with a half-life of 3.3 min, 2.8 min and 1.6 min at pH 4.6, 5.8 and 6.6, respectively. HCN and ClCN were quantitatively recovered as degradation products. For example, from 6.0 μmol of *N*-dichloroglycine of HCN and 2.7 μmol of ClCN were obtained. Total HCN (4.65 μmol) originated from 4.65 μmol of *N*-dichloroglycine (Eqn. 3), and the residual amount of *N*-dichloroglycine (1.35 μmol) was consumed for chlorination of 2.7 μmol of HCN to ClCN.

HCN as a substrate for the myeloperoxidase-H_2O_2-Cl^- chlorinating system

The continued course of glycine chlorination by myeloperoxidase in spite of HCN accumulation indicated that the chlorinating activity of myeloperoxidase was not inhibited by CN^-, and what was more, direct chlorination of HCN by myeloperoxidase-H_2O_2-Cl^- system was obtained (Table II).

Addition data on the enzymatic chlorination of HCN were obtained by tracing changes in the Soret and Visible maxima of myeloperoxidase during the reaction. Hydrogen peroxide in the concentration employed affected only slightly the spectrum of the myeloperoxidase-CN^- complex (Fig. 1A), but in the presence of Cl^- it caused complete disappearance of myeloperoxidase-CN^- derivative (Fig. 1B). This implied that Cl^- influenced the binding of CN^- by the enzyme.

The dissociation constants of the myeloperoxidase-CN^- complex estimated by the spectrophotometric titration of myeloperoxidase with CN^- were $2.5 \cdot 10^{-6}$—$1.15 \cdot 10^{-5}$ M within the pH range 6.2 to 3.4. In turn, titration of the myeloperoxidase-CN^- complex with Cl^- caused formation of the myeloperoxidase-Cl^- complex [4] (Fig. 2). The Cl^- concentration causing 50% saturation of myeloperoxidase $[Cl^-]_{50\%}$ in the presence of different concentration of CN^- showed the competitive nature of the Cl^- and CN^- bindings at the active site of myeloperoxidase (Table III). The higher the pH the lower was the apparent affinity of Cl^- to myeloperoxidase (Table III).

The results indicate that neutrophils containing myeloperoxidase and all substrates (Cl^-, glycine and H_2O_2) may be the source of CN^- in organisms,

TABLE II

CHLORINATION OF HCN BY THE MYELOPEROXIDASE-H_2O_2-Cl^- SYSTEM

Complete samples (2 ml) contained: 1 μmol NaCN, 200 μmol NaCl, 10 μg myeloperoxidase, in 50 mM citrate/phosphate buffer; at pH 5.05, 1.8 μmol H_2O_2 and at pH 4.1, 7.2 μmol H_2O_2 was added.

pH	Sample composition	After 2 min of incubation	
		HCN (μmol)	ClCN (μmol)
5.05	Complete	0.60	0.33
	—NaCl	0.95	0.00
	—H_2O_2	1.0	0.00
	—Enzyme	0.98	0.00
4.1	Complete	0.1	0.85
	—NaCl	0.98	0.00
	—H_2O_2	1.05	0.00
	—Enzyme	1.05	0.00

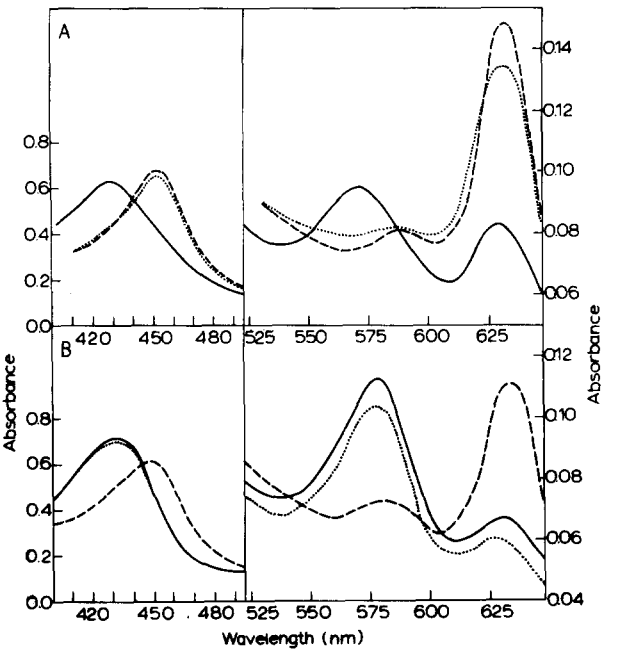

Fig. 1. The effect of Cl^- on the ability of the myeloperoxidase-CN^- complex to react with H_2O_2. (A) The absorption spectra: 6.8 μM myeloperoxidase in 50 mM phosphate/citrate buffer (pH 4.6) (———); +250 μM NaCN (- - - - - -); +300 μM H_2O_2, after 5 min and after a further 10 min (· · · · · ·). (B) The same as A, in the presence of 90 mM NaCl. Cuvette path length 1 cm.

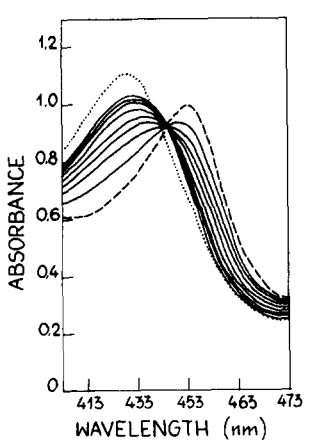

Fig. 2. Titration of the myeloperoxidase-CN^- complex with Cl^-. Myeloperoxidase, 3.15 μM (· · · · · ·) +32 μM NaCN (- - - - - -) (pH 4.56) was titrated with 2.0 M NaCl to obtain final concentrations: 6.08, 12.1, 18.1, 24.1, 35.9, 47.6, 59.2 and 70.6 mM (successive solid lines respectively). Cuvette path length 4 cm. For calculation of $[Cl^-]_{50\%}$ the dilution effect was taken into account.

TABLE III

THE EFFECT OF CYANIDE CONCENTRATION AND pH ON $[Cl^-]_{50\%}$ VALUES

pH	$[HCN] \times 10^5$	ΔA_{max}* at 431 nm	$[Cl^-]_{50\%} \times 10^2$
4.50	3.2	0.46	2.7
4.56	6.06	0.50	5.7
4.56	30.3	0.50	35
3.40	6.26	0.42	0.4
5.65	6.26	0.56	>60

* Maximal spectral difference at 431 nm between myeloperoxidase-Cl^- and nyeloperoxidase-CN^- complexes obtained from Lineweaver-Burke plot.

especially during phagocytosis, when production of hydrogen peroxide is triggered.

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