BIOORGANIC CHEMISTRY 16, 352–363 (1988)

Enzymatic Bromination of Barbituric Acid and Some of Its Derivatives

M. C. R. FRANSSEN, J. D. JANSMA, H. C. VAN DER PLAS, E. DE BOER,* AND R. WEVER*

*Laboratory of Organic Chemistry, Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands, and *Laboratory of Biochemistry, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands*

Received June 30, 1987

Barbituric acid, 1-methyl- and 1,3-dimethylbarbituric acid, some of its 5-phenyl derivatives, and 5-chlorobarbituric acid are presented as new substrates for the bromoperoxidase isolated from the brown alga *Ascophyllum nodosum*. This enzyme is able to convert these substrates into the corresponding 5-bromo or 5,5-dibromo derivatives in good yields. Kinetic measurements show that the structure of the examined substrates has little or no effect on the enzymatic rate of bromination. However, at low substrate concentration the reaction rate depends on both the concentration of the organic substrate and the concentration of hydrogen peroxide. A mechanism is proposed for the reactions of bromoperoxidase with its substrates. These reactions involve the formation of free hypobromous acid which can either brominate the organic halogen acceptor or produce singlet oxygen by a competing reaction with hydrogen peroxide.

INTRODUCTION

Haloperoxidases are enzymes capable of halogenating several organic substrates using hydrogen peroxide and halide anions (*1*). Three different types of haloperoxidases are recognized, depending on the halide ion they are able to oxidize: chloroperoxidases, bromoperoxidases, and iodoperoxidases. Thus, a chloroperoxidase oxidizes Cl^-, Br^-, and I^- ions, a bromoperoxidase oxidizes bromide and iodide ions, and an iodoperoxidase can only act on iodide ions. The chloroperoxidase from *Caldariomyces fumago* is one of the best-studied enzymes in this field; its biochemistry is well explored (*1, 2*) as well as its application to synthetic organic chemistry (*1, 3–5*). Several bromoperoxidases have been isolated, e.g., from the eggs of the sea urchin (ovoperoxidase (*6*)), from green algae (*7–9*), red algae (*9–12*), and bacteria (*13–16*), and, thus, are much more common than thought in the past. Most of these enzymes feature a heme–protein structure. Recently a novel non-heme-containing bromoperoxidase was detected in the brown alga *Ascophyllum nodosum* (*17, 18*). It was found that the isolated enzyme contained vanadium as a prosthetic group and exhibited an unusual stability for an oxidoreductase (*19, 20*). The enzyme was found to brominate monochlorodimedon (MCD)[1] and the dye phenol red. We report in this paper the results

[1] Abbreviations used: MCD, monochlorodimedon; BPO, bromoperoxidase.

0045-2068/88 $3.00

of our study on the bromination of barbituric acid (**1a**) and some of its derivatives (**1b–1f**) by this enzyme and on the kinetics of the enzymatic conversions.

MATERIALS AND METHODS

General. Bromoperoxidase (BPO) from *A. nodosum* was isolated as described previously (*18, 19*) and had a specific activity of 8–10 μmol MCD · mg protein^{-1} · min^{-1} depending on the batch used. Protein concentrations were determined by the Lowry method (*21*) using bovine serum albumin as standard. Monochlorodimedon was purchased from Sigma. 1,3-Dimethylbarbituric acid (**1c**) was obtained from Fluka and the other alkyl- and arylbarbituric acids (**1b, 1d–1e**) were synthesized from the corresponding malonic ester and urea derivatives according to the procedure of Dickey (*22*). **1f** was prepared by chlorination of barbituric acid (*23*). An authentic sample of 5,5-dibromobarbituric acid (**3a**) was obtained using Bock's method (*24*). All chemicals were of the highest commercial grade.

Ultraviolet spectra and kinetic measurements were performed on an Aminco-Chance DW-2 split-beam spectrophotometer or on a Varian DMS-100 spectrophotometer equipped with a DS-15 data station, using the Varian programs Kinetics Storage DMS-100/DS-15 (85-100541-00) and Enhanced Kinetics Calculations (85-100542-00). Initial velocities were determined by the direct linear plot method, and K_m^{app} values by means of Hanes-Woolf plots (*25*). Mass spectra were recorded on an AEI MS-902 instrument or a VG-Micromass 7070 F apparatus (direct probe mode). Circular dichroism spectra were run on a Jobin-Yvon Auto-Dichrograph Mark V. HPLC analysis was carried out on a Varian 5000 instrument (see below for experimental details).

HPLC measurements. The reactions of BPO with barbituric acid and its derivatives were monitored by HPLC as described earlier for chloroperoxidase (*4*). However, the eluent was slightly modified for the bromobarbituric acids: 15% methanol and 85% water containing 10 mM potassium phosphate and 5 mM nonyltrimethylammonium bromide, pH 7.3. The eluent flow was 1.5 ml/min. The compounds were detected by means of their uv absorption at 254 nm.

Synthesis of 5-bromobarbituric acid (***2a***). This reference compound was prepared from the phenyl iodonium betaine of barbituric acid (*26*), in a modified procedure. This betaine (0.8 g) was dissolved in a mixture of 5 ml of ethanol, 1 ml of concentrated HBr, and 6 ml of hexane. This two-phase system was stirred vigorously for 30 min at 50°C. The mixture was cooled and stored overnight at 5°C. The white precipitate formed was dried *in vacuo* over phosphorus pentoxide. The yield of **2a** was 0.43 g (2.07 mmol, 86%), mp 210–215°C (dec.), lit. 212–215°C (*24*), 205–207 (*26*). *Elemental anal.* Calcd for $C_4H_3N_2O_3Br$: C, 23.21%; H, 1.46%. Found: C, 22.94%, H, 1.60%.

Kinetic measurements. Specific activities of the enzyme were determined under the standard assay conditions (*18*): 100 mM KH_2PO_4/K_2HPO_4, pH 6.5, 100 mM KBr, 2.0 mM H_2O_2, 50 μM MCD, 25°C. The reaction was started by the addition of 8.4 nM BPO and the absorption at 293 nm was followed. These conditions were also used in all other kinetic measurements and in the experiments, where isola-

tion of the product was not required. All measurements were carried out in triplicate. The following molar extinction coefficients ($\Delta\varepsilon$, $M^{-1} \cdot cm^{-1}$) were determined at pH 6.5: MCD (21,150 at 293 nm), 5-phenylbarbituric acid (**1d;** 15,300 at 268 nm), 1-methyl-5-phenylbarbituric acid (**1e;** 15,500 at 268 nm), and 5-chlorobarbituric acid (**1f;** 11,600 at 269 nm). These data were used in all kinetic measurements.

Circular dichroism. The possible stereochemical conversion of 1-methyl-5-phenylbarbituric acid by BPO was studied in the following way. To 100 μM 1-methyl-5-phenylbarbituric acid (**1e**) in 100 mM KH_2PO_4/K_2HPO_4, pH 6.5, containing 100 mM KBr was added 2 mM H_2O_2. The reaction was monitored with a uv spectrophotometer at 268 nm after addition of 68 nM BPO to this solution. When the reaction was complete, a CD spectrum was recorded between 230 and 350 nm. A reference spectrum was made of the same solution to which buffer was added instead of H_2O_2 to correct for the CD signal of BPO itself. This spectrum was subtracted from the first one, yielding the final CD spectrum of the enzymatically synthesized product.

Isolation of enzymatic products. To prove the structure of the enzymatic products they were isolated and analyzed by mass spectroscopy. Since 5-bromobarbituric acids are too water soluble, their isolation by direct extraction with organic solvents is not very effective; therefore the bromination reaction was performed in the following way. The standard reaction medium was replaced by 0.1 M acetate buffer, pH 4.5, containing 1 mM **1,** 20 mM KBr, and 2.4 mM H_2O_2 and a suitable amount of BPO (0.17 μM) was added to the solution. Upon reaching maximal uv absorption at 268 nm, the reaction mixture was quickly frozen in liquid nitrogen and lyophilized. The 5-monobromobarbituric acids were extracted from the solid material with distilled methanol.

5,5-Dibromobarbituric acids could be easily isolated from the standard enzymatic medium by extraction with distilled ethyl acetate.

The mass spectra of all compounds were identical to those of authentic specimen. 5-Bromo-5-chlorobarbituric acid (**2f**) has not been synthesized before; its mass spectrum shows peaks at *m/e* 244 (3%), 242 (12), 240 (8), 201 (8; –HNCO), 199 (30), 197 (23), 158 (30; –HNCO), 156 (100), 154 (74), 130 (2; –CO), 128 (8), 126 (6). *Anal.* Calcd for $C_4H_2N_2O_3{}^{79}Br^{35}Cl$: 239.8938; found: 239.8938.

RESULTS

Reaction of Ascophyllum nodosum BPO with Barbituric Acid and Its Derivatives

When barbituric acid (**1a**) was incubated with BPO under standard conditions, a rapid change in the uv spectrum occurred (see Fig. 1). During the first 2½ min the original absorption peak of **1a** at 258 nm shifted to a higher wavelength (268 nm) with an isosbestic point at 266 nm. After this period the peak at 268 nm disappeared, indicating the consecutive formation of a second product (peak at 232 nm, isosbestic point at 285 nm). The uv spectra of the products perfectly match those

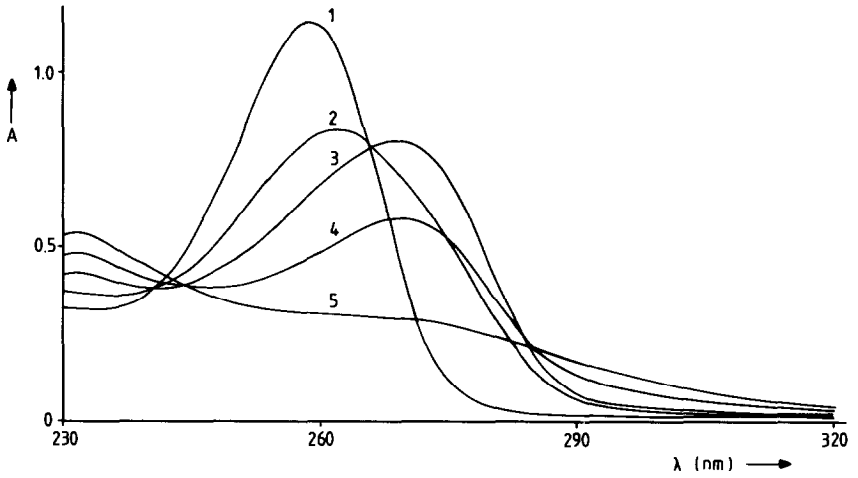
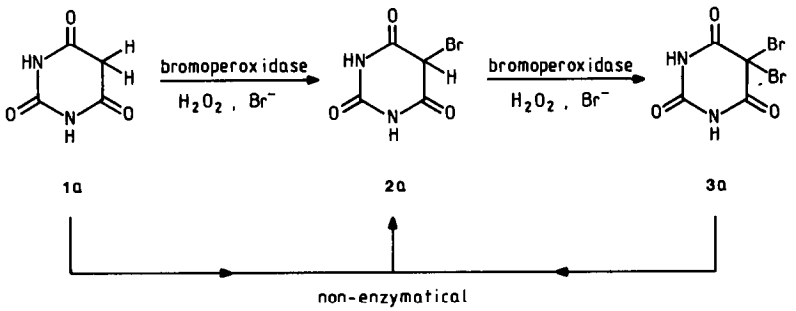


FIG. 1. Ultraviolet-absorption spectra, as recorded during the reaction of barbituric acid (**1a**) with the bromoperoxidase from *Ascophyllum nodosum*. The quartz cuvette was filled with 50 μM **1a**, 2 mM H_2O_2, 100 mM KBr, and 100 mM KH_2PO_4/K_2HPO_4, pH 6.5. To this, 2.8 nM bromoperoxidase was added and uv spectra were recorded at appropriate time intervals. Trace 1, $t = 0$ min (pure **1a**); trace 2, $t = 1.0$ min; trace 3, $t = 2.6$ min; trace 4, $t = 4.2$ min; trace 5, $t = 6.6$ min (reaction is complete).

from authentic samples of 5-bromobarbituric acid (**2a**) and 5,5-dibromobarbituric acid (**3a**), indicating that **1a** underwent bromination at C5. That these compounds are indeed formed was confirmed by mass spectra of **2a** and **3a**, isolated from the enzyme-mediated bromination of **1a**, which were identical to those obtained from authentic samples. When the reaction was monitored with reversed phase ion pair HPLC it became clear that these reactions were consecutive: the peak of **3a** appeared only when **1a** had quantitatively been converted into **2a**. A possible explanation for the accumulation of **2a**, namely, that the rate of the reaction **1a**→**2a** is much higher than that of **2a**→**3a**, is not very likely since we found that the initial rates of bromination of **1a** and **2a** are about equal. It was published recently that **3a** is a brominating agent (*27*) and this must be taken into account when studying the kinetics of this reaction. When equal amounts of **1a** and **3a** were mixed only **2a** was found on analysis of the reaction mixture by means of uv spectroscopy and HPLC. So **2a** is formed enzymatically as well as nonenzymatically; the conversion **2a**→**3a** is exclusively enzyme-mediated. This was proven by the fact that authentic **2a** was converted by the enzyme into **3a** under standard conditions. The reaction sequence is depicted in Scheme 1.

The enzymatic bromination reaction has also been investigated with substituted barbituric acids as substrates. 1-Methylbarbituric acid (**1b**) and 1,3-dimethylbarbituric acid (**1c**) were converted via their 5-bromo derivatives **2b** and **2c** into the 5,5-dibromo compounds **3b** and **3c** (see Scheme 2). The 5-phenylbarbituric acids **1d** and **1e** were also very good substrates for the enzyme and gave the corresponding 5-bromo derivatives **2d** and **2e**, respectively. Interestingly, 5-chlorobarbituric acid (**1f**) was converted to 5-bromo-5-chlorobarbituric acid (**2f**), showing the potential-



SCHEME 1. Reaction pattern of the bromination of barbituric acid (**1a**) by the bromoperoxidase from *Ascophyllum nodosum*, H_2O_2, and bromide ions. The reaction between **1a** and **3a** proceeds nonenzymatically.

ity of this procedure to synthesize compounds with two different halogen atoms attached to the same carbon atom. As can be concluded from uv spectroscopy the yields of all the brominated compounds are more than 95%.

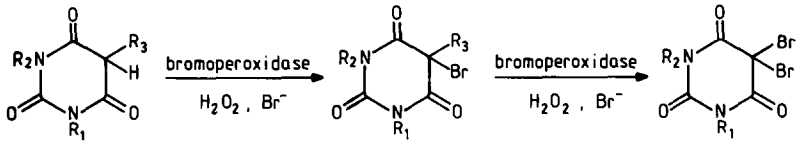
The nonenzymatic synthesis of the compounds **2a, 3a,** and **3c** have been reported. **2a** was prepared by the reaction of bromine with **1a** in water (*24*)[2] or in dimethylformamide (*28*). The latter authors reported a yield of 78%, but in our hands only undefined products were obtained. The method described by Neiland (*26*) involving the reaction of the phenyliodonium betaine of barbituric acid with hydrobromic acid is the only one which gave good results in preparing **2a,** although we found that the addition of some hexane to the reaction medium in this conversion is essential. The reason for this is not clear; perhaps the reaction is facilitated by the fact that iodobenzene, one of the products, is removed from the reaction equilibrium by the organic solvent. Another possibility is that the presence of hexane increases the solubility of one of the starting materials. **3a** and **3c** were synthesized from the corresponding violuric acids and molecular bromine in yields of about 85% (*29*). When these compounds were treated with ammonia, the 5-monobromo compounds were obtained in yields of 50%. Later syntheses of **3a** all used molecular bromine as reagent (*24*, yield not given; *27*, yield 92%).

Kinetics of the BPO-Mediated Bromination of Barbituric Acids and Monochlorodimedon

In contrast to the chloroperoxidase from *C. fumago,* the mechanism of the reaction between appropriate substrates and the *A. nodosum* bromoperoxidase has hardly been studied. As a first step in the elucidation of this mechanism we present some kinetic data on the bromination of MCD and barbituric acid (**1a**) and its derivatives **1b–1f.**

Comparison of the enzymatic bromination rate of MCD with those determined with **1d, 1f,** and **2a** at pH 4.5 or 6.5 shows that the rates of the four reactions differ only to a very small extent (see Table 1). Also the presence of methyl groups on

[2] The reported yield was 70%, but this figure could not be reproduced by us.



1 2 3

a. $R_1, R_2, R_3 = H$
b. $R_1 = CH_3$, $R_2, R_3 = H$
c. $R_1, R_2 = CH_3$, $R_3 = H$
d. $R_1, R_2 = H$, $R_3 = C_6H_5$
e. $R_1 = CH_3$, $R_2 = H$, $R_3 = C_6H_5$
f. $R_1, R_2 = H$, $R_3 = Cl$

a. $R_1, R_2 = H$
b. $R_1 = CH_3$, $R_2 = H$
c. $R_1, R_2 = CH_3$

SCHEME 2. General reaction scheme for the bromination of barbituric acid (**1a**) and some of its derivatives by bromoperoxidase, H_2O_2, and bromide ions.

TABLE 1

Relative Reaction Rates of the Enzymatic Bromination of Various Substrates[a]

	Relative reaction rate[b]	
Compound	pH 4.5	pH 6.5
MCD	100[c]	100[d]
1d	102	107
1f	103	102
2a	99	N.D.

[a] The following conditions were used: 100 mM KH_2PO_4 buffer, pH 4.5, containing 20 mM KBr, 2.4 mM H_2O_2, and 50 μM organic substrate; or 100 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.5, containing 100 mM KBr, 2 mM H_2O_2, and 50 μM organic substrate. See Materials and Methods for further details. $n = 3$.

[b] Arbitrary units; the value for MCD was set at 100. N.D., Not determined.

[c] Corresponds to 8.0 μmol MCD · mg protein^{-1} · min^{-1}.

[d] Corresponds to 8.2 μmol MCD · mg protein^{-1} · min^{-1}.

TABLE 2

Time Needed for Complete Conversion (**1**→**3**) of Some Barbituric Acids[a]

Compound	Conversion time[b]
1a	100[c]
1b	95
1c	98

[a] Determined under standard assay conditions by monitoring the uv absorption at 270 nm. $n = 3$.

[b] Arbitrary units; the value for **1a** was set at 100.

[c] Corresponds to 145 s.

the nitrogen atoms of barbituric acid has no effect on the rate of bromination (see Table 2) as determined by the total time in which the reaction **1**→**3** is completed. However, the differences in reaction rates may in this case be masked by the nonenzymatic side reaction (*vide supra*).

These results are in full agreement with those found for chloroperoxidase: both enzymes seem to have little specificity regarding the structure of their organic substrates. Contrastingly, however, the concentration of the organic substrate has a marked influence on the bromination rate with BPO. In the range 2–50 μM the reaction rate increases in a hyperbolic fashion with increasing concentration of the organic substrate, as is shown in Fig. 2a for 5-phenylbarbituric acid (**1d**). The obtained data show a linear relationship when put in a Hanes-Woolf plot (see Fig. 2b). In this way, apparent Michaelis-Menten constants and maximal rates were obtained for MCD and **1d–1f** (Table 3). It should be noted here that strictly taken, these constants may not be considered as true Michaelis-Menten constants for the organic substrates (cf. Discussion). The maximal rates of bromination of the various substrates at high substrate concentration are practically identical. However, the K_m^{app} values varied somewhat, although they are quite independent of the structure of the rest of the molecule, being carbocyclic or heterocyclic. Also the nature of the substituent between the two carbonyl groups is of minor importance, although the presence of a 5-phenyl substituent seems to lower the interaction somewhat. However, the magnitude of the K_m^{app} values increased when more H_2O_2 was present (see Table 3).

Stereochemistry of the Bromination Reaction

To determine whether the enzyme-mediated bromination reaction shows any stereoselectivity, we investigated the bromination of 1-methyl-5-phenylbarbituric acid (**1e**). Monitoring the reaction by circular dichroism clearly showed that a racemic mixture was obtained.

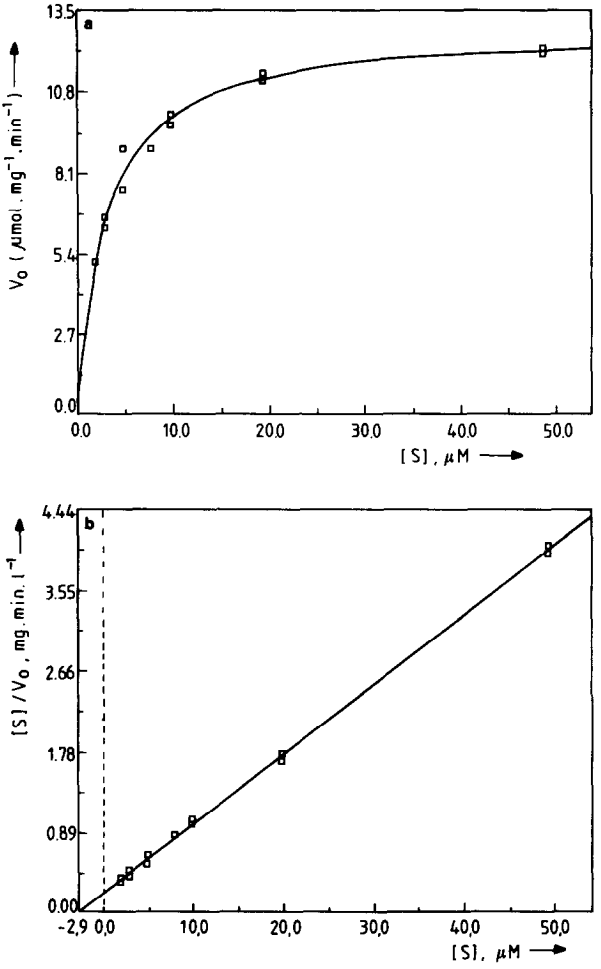


FIG. 2. (a) The effect of the concentration of **1d** on the reaction rate of bromoperoxidase. Each dot represents the mean of average values obtained from three experiments. (b) The same data used in (a), depicted in a Hanes-Woolf plot. The intercept on the [S]-axis gives the K_m^{app} values; the slope equals the reciprocal of the maximal bromination rate.

DISCUSSION

The bromoperoxidase from the brown alga *A. nodosum* converts barbituric acid and some of its derivatives smoothly into their 5-bromo or 5,5-dibromo compounds. The yields were high, so we consider our enzymatic procedure to be competitive with the chemical syntheses described above and as such the enzyme could be used for synthetic purposes. The occurrence of a nonenzymatic side reaction poses no problem in this respect and affords the isolation of the intermediate products **2a–2c** when the reaction is stopped halfway. **3a** is a smooth bromin-

TABLE 3

K_m^{app} Values and Maximal Bromination Rates for Some Bromoperoxidase Substrates at Three Different H_2O_2 Concentrations[a]

a. K_m^{app} values

Compound	$[H_2O_2]$ (mM) 0.8	2.0	3.0
MCD	1.8	4.0	4.7
1d	1.9	1.9	2.9
1e	0.6	1.6	2.3
1f	1.6	2.0	3.5

b. Maximal bromination rates

Compound	$[H_2O_2]$ (mM) 0.8	2.0	3.0
MCD	10.9	12.7	11.5
1d	13.7	13.1	12.9
1e	12.6	12.7	12.4
1f	12.1	11.6	12.4

[a] See text for experimental details. K_m^{app} values are expressed in μM and maximal bromination rates in μmole · mg protein^{-1} · min^{-1}.

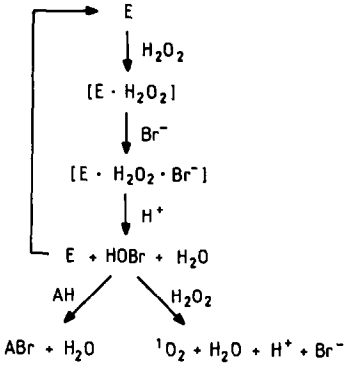
ating agent for saturated and α,β-unsaturated carbonyl compounds (*27*), which further supports the reaction pattern as shown in Scheme 1.

There is a lot of literature on the reaction mechanism of haloperoxidases, but as yet not everything is fully understood. There is agreement in the literature about lactoperoxidase and the chloride-metabolizing enzyme myeloperoxidase: both enzymes most probably form hypohalous acids (HOCl, HOBr), which are the active halogenating species in their reactions (*1*). The data concerning the chloroperoxidase from *C. fumago* are still confusing (see Ref. (*4*) for a discussion on this topic), and virtually nothing is known about the other haloperoxidases. The *A. nodosum* bromoperoxidase is unique in the sense that it is not a heme–protein, but contains a vanadium (V) ion in its active center. In addition, unlike heme-containing peroxidases, this enzyme does not oxidize electron-rich organic compounds in the presence of hydrogen peroxide (*30*). As outlined in Ref. (*31*) the vanadium (V) ion serves as a binding site for the substrate hydrogen peroxide and does not change valence state during catalytic turnover. Such a model is supported by the well-known property of inorganic vanadium (V) compounds to form stable peroxovanadium (V) complexes with hydrogen peroxide (*32*). Thus, the reaction mechanism of this enzyme must be different from that of the other haloperoxidases. In the work presented here it is shown that the enzyme closely resembles chloro-

peroxidase with respect to substrate specificity and stereochemical behavior (*4*): (i) in the case of cyclic β-dicarbonyl compounds the enzymatic activity is not affected by the structure of the ring being carbocyclic or heterocyclic; (ii) the enzymatic reaction rate is not affected by the presence of methyl or phenyl substituents attached to the substrate barbituric acid; (iii) 1-methyl-5-phenylbarbituric acid (**1e**) is not halogenated in a stereospecific manner. However, for chloroperoxidase no hyperbolic relation between the rate of chlorination and the concentration of the organic substrate could be found, whereas for the bromoperoxidase studied here the concentration of the organic substrate has a marked influence on the bromination rate, yielding K_m^{app} values in the range 0.6–5 μM (see Table 3). That the nature of the various organic substrates does not largely affect the rate of bromination suggests that under our experimental conditions, formation of a rate-limiting quaternary complex between a halogenating enzyme intermediate and an organic substrate does not occur. Apparently some step earlier in the reaction mechanism is rate-limiting. However, at low substrate concentrations the bromination rate is a function of the substrate concentration. Although the latter may indicate the existence of such a quaternary complex, it should be noted that the K_m^{app} values increase at higher concentrations of H_2O_2. This rules out an ordered *ter* mechanism, because in that case the concentration of H_2O_2 should have no influence on the K_m^{app} values. Therefore, it is more likely that we deal with a reaction in which the enzyme produces free HOBr. The hypobromous acid formed may then react with the organic substrate or in a competitive reaction with H_2O_2 to give singlet oxygen (*33*). Thus, at an increased concentration of H_2O_2 there is less HOBr available for the organic substrate. As a consequence the apparent Michaelis-Menten constants rather reflect the affinity between HOBr and the organic substrate relative to the affinity between HOBr and H_2O_2 and cannot be considered as true Michaelis-Menten constants for the substrates. This suggestion explains why at higher H_2O_2 concentrations the K_m^{app} values increase (Table 3). The experimental conditions were such that all enzyme molecules are saturated with H_2O_2 and Br^- (*18*). This explains why, at higher concentrations of the organic substrates, the maximal bromination rate is not affected by H_2O_2. This phenomenon is not observed in chloroperoxidase-catalyzed reactions, probably because of differences in experimental conditions. The experiments reported here were performed at pH 6.5, whereas the studies on chloroperoxidase were performed at pH 2.7, the pH optimum of this enzyme. As shown by Kanofski (*34*) the rate of the reaction between HOBr and H_2O_2 at neutral pH is very high, whereas it is low at acid pH (*35*). This is substantiated by our observation that at pH 4.5 the K_m^{app} values for the BPO-mediated bromination of MCD decreased considerably.

We have to conclude therefore that at pH 6.5 the rate of the reaction of HOBr with H_2O_2 is of the same magnitude as that between our organic substrates and HOBr. The small differences in the K_m^{app} values of the various substrates may reflect differences in rate constants of the reaction between HOBr and the various organic substrates. Thus our experimental data favor a mechanism (see Scheme 3) in which bromoperoxidase generates free hypobromous acid, although we cannot rule out a short-lived enzyme-bound halogenating intermediate.

That **1e** is in fact not brominated stereoselectively is due to the brominating



SCHEME 3. Tentative reaction mechanism of the bromoperoxidase-induced bromination of organic compounds. E, Bromoperoxidase from *Ascophyllum nodosum;* AH, organic substrate; ABr, organic product.

properties of compounds like **3** and **2d, 2e** themselves which in fact nonstereoselectively introduce bromo atoms into the organic substrates in solution, giving a racemic mixture.

Itoh *et al.* (*36*) recently reported on the bromination of nucleobases by a nonheme bromoperoxidase from *Corallina pilulifera*. Some of the heterocyclics were brominated by the enzyme but not by molecular bromine. The authors therefore concluded that these substrates were brominated at the active site of the enzyme, which indicates that the reaction mechanism of the *Corallina* bromoperoxidase may differ from that of our enzyme.

The bromoperoxidase from *A. nodosum* is an interesting enzyme, because of its unique structure, its selectivity for bromide, and its remarkable stability toward organic solvents (*18, 20*). In this study we showed that the enzyme is a smooth brominating agent because it produces HOBr in a very low concentration. Therefore it will be studied more extensively in the near future.

ACKNOWLEDGMENTS

We are much indebted to Dr. M. A. Posthumus, Dr. C. A. Landheer, Mr. C. J. Teunis, and Mr. H. Jongejan for carrying out mass spectroscopic measurements and elemental analysis. We gratefully acknowledge the support by Naamloze Vennootschap DSM, Heerlen, The Netherlands. This work is part of the research program of the Netherlands Foundation for Chemical Research (SON) and was made possible by financial support from the Netherlands Technology Foundation (STW).

REFERENCES

1. NEIDLEMAN, S. L., AND GEIGERT, J. (1986) Biohalogenation: Principles, Basic roles and Applications, pp. 1–203, Ellis Horwood, Chichester.
2. HAGER, L. P., MORRIS, D. R., BROWN, F. S., AND EBERWEIN, H. (1966) *J. Biol. Chem.* **241,** 1769.
3. FRANSSEN, M. C. R., AND VAN DER PLAS, H. C. (1984) *Recl. Trav. Chim. Pays-Bas* **103,** 99.

4. FRANSSEN, M. C. R., AND VAN DER PLAS, H. C. (1987) *Bioorg. Chem.* **15,** 59.
5. FRANSSEN, M. C. R., VAN BOVEN, H. G., AND VAN DER PLAS, H. C. (1988) *J. Heterocyclic Chem.* **24,** 1313.
6. DEITS, T., FARRANCE, M., KAY, E. S., MEDILL, L., TURNER, E. E., WEIDMAN, P. J., AND SHAPIRO, B. M. (1984) *J. Biol. Chem.* **259,** 13525.
7. BADEN, D. G., AND CORBETT, M. D. (1980) *Biochem. J.* **187,** 205.
8. MANTHEY, J. A., AND HAGER, L. P. (1981) *J. Biol. Chem.* **256,** 11232.
9. YAMADA, H., ITOH, N., MURAKAMI, S., AND IZUMI, Y. (1985) *Agric. Biol. Chem.* **49,** 2961.
10. AHERN, T. J., ALLAN, G. G., AND MEDCALF, D. G. (1980) *Biochim. Biophys. Acta* **616,** 329.
11. ITOH, N., IZUMI, Y., AND YAMADA, H. (1986) *J. Biol. Chem.* **261,** 5194.
12. KRENN, B. E., PLAT, H., AND WEVER, R. (1987) *Biochim. Biophys. Acta* **912,** 287.
13. VAN PÉE, K.-H., AND LINGENS, F. (1984) *FEBS Lett.* **173,** 5.
14. WIESNER, W., VAN PÉE, K.-H., AND LINGENS, F. (1985) *Biol. Chem. Hoppe-Seyler* **366,** 1085.
15. VAN PÉE, K.-H., AND LINGENS, F. (1985) *J. Bacteriol.* **161,** 1171.
16. VAN PÉE, K.-H., AND LINGENS, F. (1985) *J. Gen. Microbiol.* **131,** 1911.
17. VILTER, H. (1984) *Phytochemistry* **23,** 1387.
18. WEVER, R., PLAT, H., AND DE BOER, E. (1985) *Biochim. Biophys. Acta* **830,** 181.
19. DE BOER, E., VAN KOOYK, Y., TROMP, M. G. M., PLAT, H., AND WEVER, R. (1986) *Biochim. Biophys. Acta* **869,** 48.
20. DE BOER, E., PLAT, H., TROMP, M. G. M., WEVER, R., FRANSSEN, M. C. R., VAN DER PLAS, H. C., MEIJER, E. M., AND SCHOEMAKER, H. E. (1987) *Biotechnol. Bioeng.* **30,** 607.
21. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
22. DICKEY, J. B., AND GRAY, A. R. (1938) *Org. Syn.* **18,** 8.
23. BOCK, W. (1923) *Ber. Dtsch. Chem. Ges.* **56,** 1222.
24. BOCK, W. (1922) *Ber. Dtsch. Chem. Ges.* **55,** 3400.
25. SEGEL, I. H. (1975) Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 1–957, Wiley, New York.
26. NEILAND, O. YA., AND NEIMAN, D. E. (1970) *J. Org. Chem. USSR* **6,** 2522.
27. GRUNDKE, G., KEESE, W., AND RIMPLER, M. (1985) *Chem. Ber.* **118,** 4288.
28. GRANADOS, R., MARQUEZ, F., AND MELGAREJO, M. (1962) *Anales Real Soc. Españ. Fis. Quim. Ser. B* **58,** 479; *Chem. Abstr.* (1963) **59,** 16316.
29. BILTZ, H., AND HAMBURGER, T. (1916) *Ber. Dtsch. Chem. Ges.* **49,** 635.
30. DE BOER, E., PLAT, H., AND WEVER, R. (1987) *in* Biocatalysis in Organic Media (Laane, C., Tramper, J., and Lilly, M. D., Eds.), Elsevier, Amsterdam; *Stud. Org. Chem.* **29,** 317.
31. DE BOER, E., BOON, K., AND WEVER, R. (1988) *Biochemistry,* **27,** 1629.
32. MIMOUN, H., SAUSINNE, J., AND WEISS, R. (1983) *J. Amer. Chem. Soc.* **105,** 3101.
33. KANOFSKI, J. R. (1984) *J. Biol. Chem.* **259,** 5596.
34. KANOFSKI, J. R. (1986) *Biochem. Biophys. Res. Commun.* **134,** 777.
35. LIBBY, R. D., THOMAS, J. A., KAISER, L. W., AND HAGER, L. P. (1982) *J. Biol. Chem.* **257,** 5030.
36. ITOH, N., IZUMI, Y., AND YAMADA, H. (1987) *Biochemistry* **26,** 282.