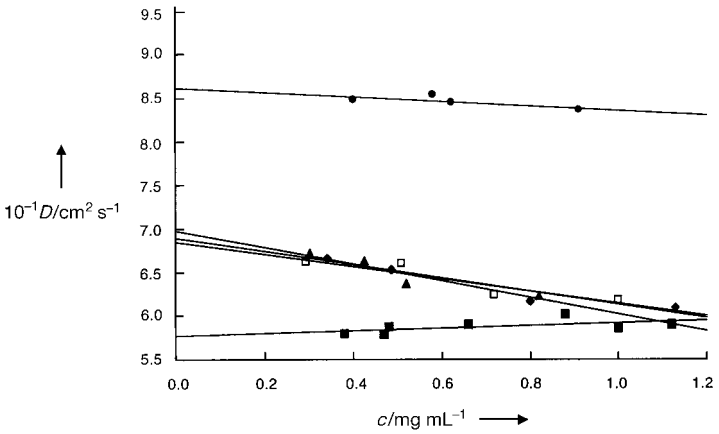

Figure 1. Variation of the translational diffusion coefficient $D_T(c)$ as a function of concentration c of GA (■), CD (●), GA + **1** (◆), GA + **2** (▲), and GA + **3** (□).

The extrapolated values at infinite dilution gave hydrodynamic sizes of 78.5 Å (major semi-axis) and 30 Å (semi-axis), for the GA and CD, respectively. These values are in good agreement with those obtained previously with other techniques (141 and 60 – 65 Å for the major and minor axis of GA, respectively).[5, 6] The diffusion coefficients for **1**, **2**, and **3** in the presence of GA show a decrease as a function of concentration (Figure 1).

The three systems gave roughly the same concentration dependence within experimental errors. The associated diffusion coefficients decrease with the protein concentration and the extrapolated value at infinite dilution gave a hydrodynamic size of 62 Å (major semi-axis). However a value of 72.5 Å was found with the longer probe **4**. These values are intermediate between systems with a CD and GA only; and is in fact expected if the ligand somehow decreases the size of the GA system. The negative value of the slope of D_T versus c shows that there is a strong attraction between the particles and reflects the thermodynamics of the system; in general the value of the slope is pH dependent. It appears from these results that the presence of the bound bifunctional ligands stabilizes a more compact conformation of GA. If this motion of the two domains also occurs upon binding of the natural substrate it may achieve the processivity of this exo-enzyme: GA would bind to the polymer through its SBD and the motion of the CD would allow the cleavage of several glucose moieties at the nonreducing end of the same glucan chain before the release of the enzyme. This new concept is under investigation by using mutants of glucoamylase with linkers of various lengths.

Experimental Section

All compounds were homogeneous according to elemental analysis or HR-MS and ^{1}H and ^{13}C NMR spectra.

Dynamic light scattering experiments: Sample preparation, equipment, and data analysis are essentially the same as described in ref. [9], except that sodium acetate buffer (50 mM, pH 4.2) and a temperature of 27 °C were used. Equimolar ratios of ligands and enzyme were utilized.

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Metal-Free Haloperoxidases: Fact or Artifact?

Ole Kirk* and Lars Sparre Conrad

Haloperoxidases are enzymes that catalyze the oxidation of a halide ion (X^-) to the corresponding hypohalous acid [Eq. (1)]. The hypohalous acid produced can further react with different nucleophilic acceptors to form a diversity of halogenated compounds.[1, 2] Different classes of haloperox-

$$H_2O_2 + X^- + H^+ \overset{\text{haloperoxidase}}{\rightleftharpoons} HOX + H_2O \qquad (1)$$

[*] Dr. O. Kirk
Department of Bio-Organic Chemistry
Enzyme Research, Novo Nordisk A/S
Novo Alle, DK-2880 Bagsvaerd (Denmark)
Fax: (+45) 4442-3206
E-mail: oki@novo.dk
Dr. L. Sparre Conrad
Department of Formulation Design
Enzyme Development and Application, Novo Nordisk A/S
Novo Alle, 2880 Bagsvaerd (Denmark)

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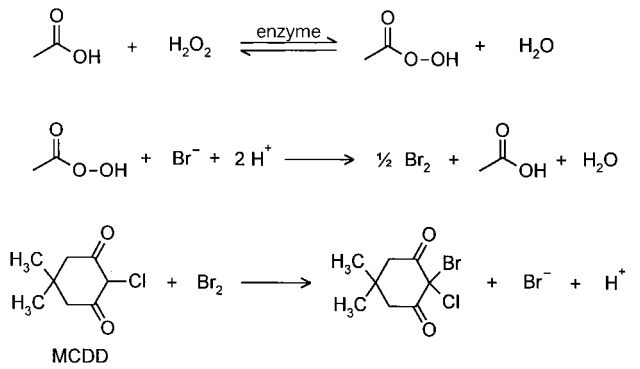
idases have been identified. Two types of haloperoxidases carry a prosthetic group: a heme group in the case of the heme-containing haloperoxidases and a vanadate ion (VO_4^{3-}) in the case of the vanadium-containing haloperoxidases.[1, 3]

Recently, a third class of haloperoxidases, the so-called metal-free haloperoxidases, was described by van Pée et al.[4] The mode of action of these haloperoxidases was unknown although a proposal was made that involved the formation of an intermediate methionine sulfoxide in the active site.[5] It was, therefore, very surprising that an X-ray structural analysis of a metal-free bromoperoxidase[6] did reveal the presence of a catalytic triad (Ser-His-Asp) commonly found in lipases, esterases, and serine proteases.[7] Combined with the fact that these enzymes exhibit activity only in acetate buffer, a mechanism was suggested by Berkessel, Krauss, van Pée, and co-workers[8] in which the halogenation of monochlorodimedone (2-chloro-5,5-dimethyl-1,3-cyclohexadione, MCDD) in the presence of acetate, H_2O_2, Br^-, and the enzyme is explained by the reaction sequence outlined in Scheme 1.



Scheme 1. The mechanism of MCDD halogenation when catalyzed by a "metal-free haloperoxidase". A low stationary-state concentration of peracetic acid is formed by perhydrolysis of acetic acid.[8]

The enzyme-catalyzed step in the process is perhydrolysis (that is, lysis with hydrogen peroxide) of acetic acid to form peracetic acid. Unfortunately, the authors were not able to prove the presence of peracetic acid in their reactions because of a lack of sufficiently sensitive analytical tools. An indirect proof was, however, obtained when the metal-free haloperoxidase, which was also found to have esterase activity towards 4-nitrophenyl acetate, was found to degrade solutions of pre-formed peracetic acid. Four proteases as well as a choline esterase were also tested for haloperoxidase activity under the same experimental conditions by the authors, but only choline esterase gave a positive reaction. It was, therefore, concluded that the ability to catalyze perhydrolysis is not a general characteristic of enzymes with a catalytic mechanism based on a Ser-His-Asp catalytic triad, and that a novel enzymatic system had been introduced.

We have described previously a highly sensitive assay based on HPLC with electrochemical detection for the detection of peracids in complex mixtures.[9] This system was, furthermore, used to monitor the ability of different hydrolases to catalyze both the degradation and formation of peracids.[10] It could be concluded from this study that many different hydrolases with a Ser-His-Asp catalytic triad are, indeed, able to catalyze perhydrolysis of relevant substrates. Thus, lipases were found to catalyze perhydrolysis of long-chain carboxylic acids while only esterases, including choline esterase, were able to catalyze perhydrolysis of acetic acid. These results somewhat contradict the proposal made by Berkessel et al. that the ability to catalyze perhydrolysis is an unusual activity. The aim of the present study was, therefore, to investigate if another class of enzymes, lipases, in general would be able to give a positive haloperoxidase reaction under the experimental conditions of Berkessel et al. and, if this was the case, to investigate if the expected peracid was formed during the course of the reaction.

As we have found previously (see above) that lipases are capable of catalyzing perhydrolysis of long-chain acyl substrates the experimental setup described by Berkessel et al. was adjusted by substituting the 1M acetate buffer used with a hexanoic acid buffer. This buffer had to be only 0.2M as a consequence of the more limited solubility of hexanoic acid. Three lipases derived from *Fusarium oxysporum*, *Humicola lanuginosa*, and *Candida antarctica* (B-component) were selected for the study. As indicated in Figure 1 both the

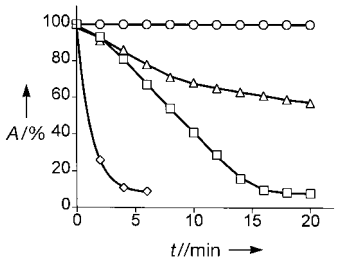


Figure 1. Profile of MCDD halogenation catalyzed by lipases. ○ control, ◇ 100μM pre-formed peracid, □ *Fusarium* lipase, △ *Humicola* lipase.

Fusarium and the *Humicola* lipases gave a positive haloperoxidase reaction as measured by the bromination of MCDD. The *Fusarium* lipase appeared to be stable under the experimental conditions and capable of transforming all available MCDD. In contrast, the A_{290} curve for the *Humicola* lipase was only linear up to about seven minutes, while data obtained beyond this point suggests an inactivation of the enzyme. No conclusive data were, unfortunately, obtained with the *Candida* lipase as an impurity in the enzyme preparation gave rise to a strong UV absorption that interfered with the assay. All lipases were, however, able to produce perhexanoic acid under the experimental conditions used even though the concentrations, as expected, were very low (Table 1).

Table 1. Formation of peroxyhexanoic acid by using different lipase catalysts.

Lipase	$[H_2O_2]$ [mM]	[peroxyhexanoic acid][a] [μM]
Fusarium oxysporum	250	372
	50	128
	7.2	17
Humicola lanuginosa	250	194
Candida antarctica B	250	147
(no enzyme)	250	0

[a] After 15 min.

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As expected from the experiments in which the halogenation of MCDD was monitored, the *Fusarium* lipase was superior to the *Humicola* lipase with respect to peracid formation after 15 minutes. In the case of the *Fusarium* lipase, the effect of varying the concentration of hydrogen peroxide was investigated, and a clear dose–response effect was seen. The equilibrium concentration of only 17 μM measured under the conditions used for halogenation of MCDD should, however, be sufficient to account for the conversion of MCDD as this scavenger was present in a concentration of only 44 μM.

To further support the hypothesis that the peracid formed was, indeed, responsible for the observed halogenation of MCDD, pre-formed peracid was added in experiments performed without any enzyme. As indicated in Figure 1, addition of 100 μM pre-formed peracid did, as expected, result in a very rapid halogenation of MCDD. Finally, the lipase-based systems were compared to a vanadium-containing microbial haloperoxidase derived from *Curvularia verruculosa*.[11] Addition of this enzyme in a concentration of 0.4 $\mu g\,mL^{-1}$ resulted in complete halogenation of MCDD in less than two minutes, both in the hexanoate buffer and in a phosphate buffer (results not shown). No peracid could, as expected, be detected in experiments performed in the hexanoate buffer. These experiments further highlight the difference between a true haloperoxidase and the apparent "metal-free haloperoxidase" activity exhibited by a hydrolase.

Our investigations show that lipases can also exhibit metal-free haloperoxidase activity in the presence of an acyl substrate, hydrogen peroxide, and halide ions. This apparent haloperoxidase activity is shown to result from the formation of a peracid, which can be detected in very low concentrations. We therefore propose that the postulated unique enzymatic activity of metal-free haloperoxidases previously described[8] is, in fact, a common side-activity of hydrolases such as lipases and esterases.

Experimental Section

General: The lipases derived from *Fusarium oxysporum*, *Humicola lanuginosa*, and *Candida antarctica* were obtained from Novo Nordisk A/S (Denmark) and characterized as described previously.[10] The haloperoxidase from *Curvularia verruculosa* was also obtained from Novo Nordisk A/S and prepared as described by Fuglsang et al.[11] MCDD and potassium bromide were obtained from Sigma, while H_2O_2 and all other chemicals (all of analytical grade) were obtained from Merck. Reference samples of peroxyoctanoic acid and peroxyhexanoic acid were made according to literature procedures by H_2O_2 oxidation of the parent acid using acidic[12] and lipase catalysis,[13] respectively.

Assay for haloperoxidase activity*:* The experiments were performed according to the procedure described in ref. [8], but using a 0.2 M hexanoic acid buffer pH 5.5 instead of a 1M acetate buffer. In short, a typical experiment was initiated by adding 100 $LU\,mL^{-1}$ (LU = lipase unit; 1 LU is defined as the amount of lipase that liberates 1 μmol fatty acid from tributyrin under standard conditions. A detailed description of the assay is available from Novo Nordisk A/S as AF 95/5) lipase to a test solution containing MCDD (44 μM), H_2O_2 (7.2 mM), and KBr (82 mM) in the hexanoic acid buffer at 25 °C. The reaction was followed spectrophotometrically and halogenation of MCDD was evident from a decrease in absorbance at 290 nm.

HPLC assay to monitor the formation of peracid: The assay was performed as previously described[9] using HPLC with electrochemical detection, a reversed phase column (LiChrosorb RP-18, Merck) with 20 mM phosphate buffer pH 6/methanol (50/50) as the mobile phase. As the detector response of different peroxycarboxylic acids has previously been found to be independent of the peracid chain-length[9] the system was calibrated with peroxyoctanoic acid, which was available in highly pure crystalline form, while a reference sample of peroxyhexanoic acid was used to define the retention time. Experiments were performed as above, only varying the H_2O_2 concentration as indicated in Table 1 and omitting the addition of MCDD and KBr. As the very high concentration of salts gave some interference with the electrochemical detector, samples were extracted with hexane (2 mL sample extracted with 1 mL hexane) and 100 μL of the hexane phase was then diluted in 400 μL mobile phase prior to analysis. Formation of peroxyhexanoic acid was monitored after 0 and 15 min in all experiments.

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