Identification of intermediates in the catalytic cycle of chloroperoxidase

Hans-Achim Wagenknecht and Wolf-Dietrich Woggon

Background: Chloroperoxidase (CPO) is the most versatile of the heme-thiolate proteins, catalyzing the chlorination of activated C–H bonds and reactions reminiscent of peroxidase, catalase, and cytochrome P450. Despite 30 years of continuous efforts, no intermediates of the enzyme's catalytic cycle have been identified except for compound I. Thus, in the absence of conclusive evidence it is generally believed that the halogenation of substrates proceeds by means of 'free HOCl' in solution.

Results: The pH profile of chloroperoxidase from *Caldariomyces fumago* revealed a new active-site complex that can be detected only at pH 4.4. According to ultra-violet (UV) spectroscopy, and by comparison with suitable enzyme models, this intermediate is the HOCl adduct of the iron(III) protoporphyrin(IX). Inactivation of chloroperoxidase by diethyl pyrocarbonate, which interrupts the proton shuttle by modification of the distal histidine, led to the formation of the $^{-}$OCl adduct of the iron complex, which was identified by comparison with a corresponding active site analogue.

Conclusions: The availability of enzyme models of heme-thiolate proteins allowed the identification by UV spectroscopy of both the $^{-}$OCl adduct and the HOCl adduct of the iron(III) protoporphyrin(IX) of chloroperoxidase. The existence of these previously elusive intermediates suggests that the chlorination catalyzed by CPO, and its corresponding active site analogue, proceeds by Cl^{+} transfer from the HOCl adduct to the substrate bound in the distal pocket of the enzyme.

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Introduction

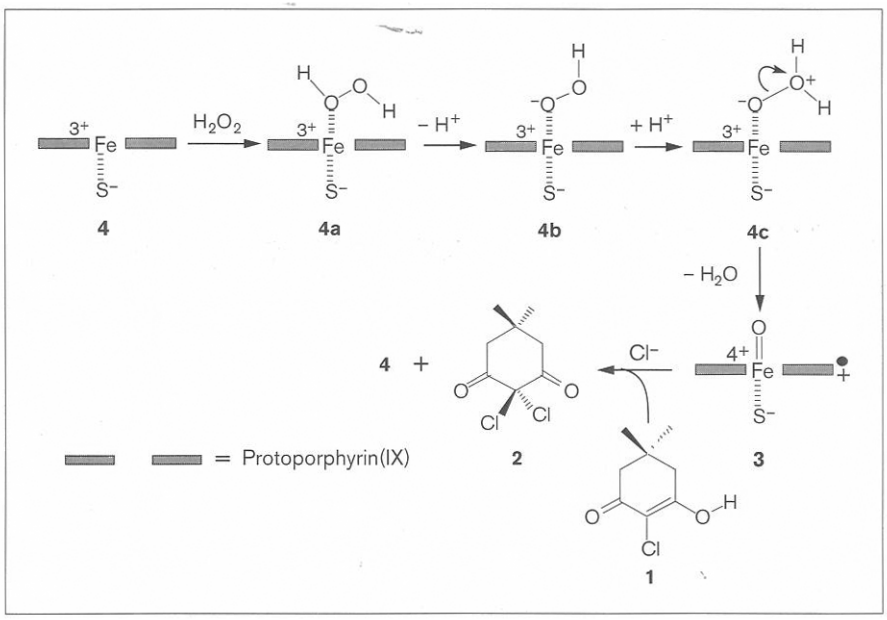
The abundant heme-thiolate proteins comprise enzymes such as cytochrome P450 [1,2], chloroperoxidase (CPO) [3–5], and nitric oxide (NO) synthase [6]. These enzymes catalyze a surprisingly large repertoire of oxidation reactions even though their active sites harbour the same centre of reactivity, an iron(III) protoporphyrin(IX) complex bound to the protein via hydrogen bridges and a thiolate that coordinates to the iron. The thiolate ligand belongs to a cysteine residue situated in a highly conserved region of the protein. Most of our knowledge regarding the reaction mechanisms of the heme-thiolate proteins relies on studies of different cytochromes P450, in particular $P450_{cam}$, and suitable synthetic enzyme models [7]. X-ray structures of various cytochromes P450 [8,9] and of CPO [10] have confirmed earlier information on the coordination chemistry of the active site. Furthermore, they revealed the distinct mode of ligand and substrate binding in the domain opposite the thiolate binding site, thus leading to a detailed picture of the substrate access channels and proton shuttle systems required for O–O bond scission.

The catalytic cycle of cytochrome P450 is reasonably well understood, except for some details concerning the low-spin character of the resting state [11] and the electronic nature of the high-valency oxo-iron intermediate which actually performs oxygen insertion, for example in nonactivated C–H bonds. In contrast, much less is known about CPO and the more recently discovered NO synthase.

CPO, first isolated over 30 years ago from the mold *Caldariomyces fumago* [12], is the most versatile of the heme-thiolate proteins. The enzyme not only catalyzes the halogenation of activated C–H bonds, but also performs reactions reminiscent of peroxidase, catalase, and cytochrome P450 [3–5].

In order to catalyze chlorination, CPO employs H_2O_2 and Cl^{-} at pH 3 to react with 1,3 diketones, such as monochlorodimedone (Figure 1, **1**) to yield 2,2-dichlorodimedone (Figure 1, **2**). It was suggested [13,14] that chlorination proceeds via compound I (an iron(IV) porphyrin in which the fifth ligand (S^{-} for CPO; Figure 1, **3**) is enzyme dependent) that is produced when the resting state of CPO (**4**) reacts with H_2O_2 (Figure 1). Nothing is known about the mechanism of the formation of CPO's compound I (**3**), but the cleavage of H_2O_2 by horseradish

Figure 1



Reaction of the resting state of CPO (**4**) with H_2O_2 to yield compound I (**3**), which then chlorinates the substrate monochlorodimedone (**1**) in the presence of Cl^-.

peroxidase (HRP) has been investigated in detail by calculations [15,16], stopped-flow kinetics [17,18], and using (R38L) HPRC, a variant of HRP [19]. These results indicate that an H_2O_2 adduct of HRP (that is analogous to **4a** (Figure 1) except that a histidine replaces the thiolate of the proximal cysteine) is a discrete intermediate. It is further suggested that the H_2O_2 adduct is deprotonated to a transient peroxo-anion species, corresponding to **4b** (Figure 1), which after protonation of the terminal oxygen (**4c**), (Figure 1) releases water to give compound I. The reprotonation sequence is assisted by histidine and arginine, two adjacent distal amino acids. It can be assumed that the overall H_2O_2 cleavage catalyzed by CPO, as tentatively drawn in Figure 1, proceeds as for HRP. Nevertheless, for CPO there are remarkably different situations with respect to the nature of amino acids near the H_2O_2 binding site (see below) and due to the presence of the weak thiolate ligand. Both effects are expected to change the kinetics of compound I formation and, in particular, the thiolate ligand is thought to trigger the spin states of intermediates, resulting in different UV spectra and electron spin resonance (ESR) spectra when compared with those of HRP.

In the case of CPO, it is thought that when **3** reacts with Cl^-, depending on the concentration of the Cl^- either Cl_2 or HOCl is released into solution and, as a result, the substance RH is halogenated. The participation of a 'free halogenating species' seems to be in agreement with the observation that the halogenation of small, apolar substances often proceeds non-stereospecifically [20,21]. In contrast, it has been shown for example, that a highly substituted glycal is 'halohydrated' completely stereospecifically by means of CPO/KBr-H_2O_2 at pH 3 [22,23]. Despite numerous investigations of CPO [11], the mechanism of chlorination and the identification of significant reactive intermediates has remained elusive.

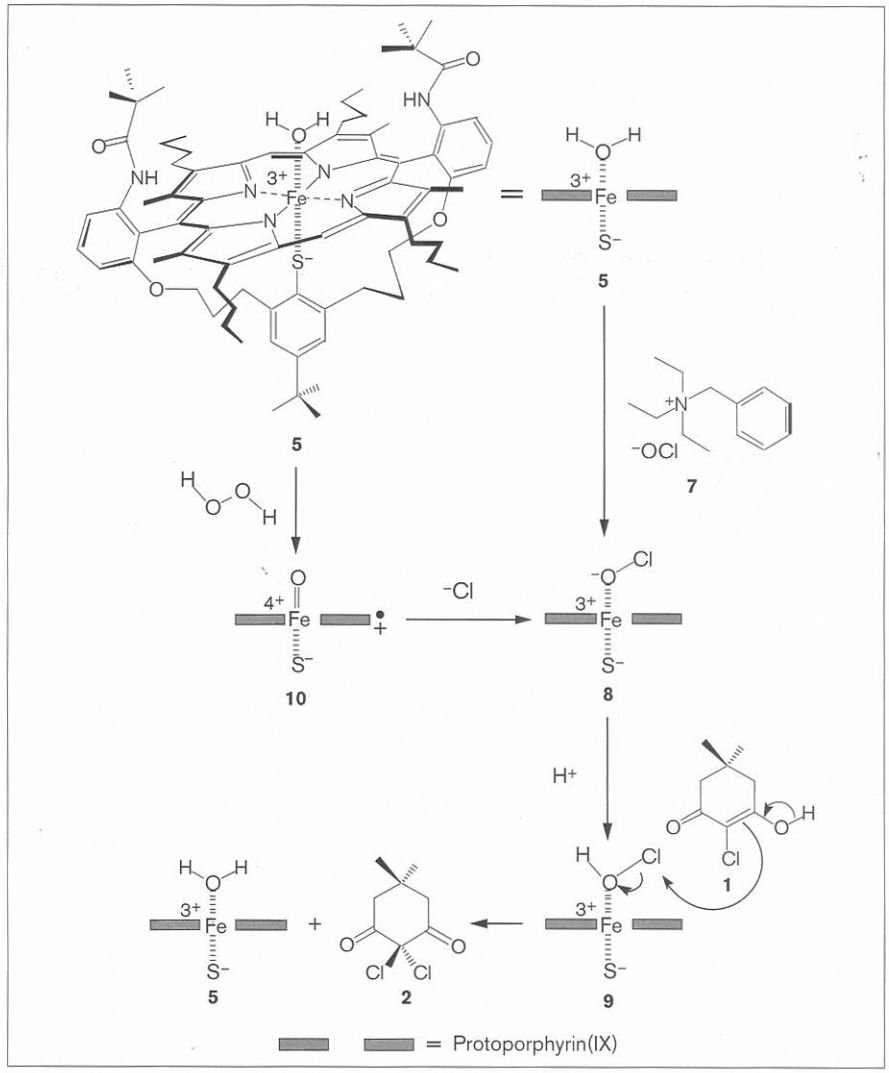
Here, we report a study aimed at identifying enzymic intermediates of CPO, guided by recent results obtained from investigating synthetic enzyme models [24].

Results and discussion

The face-protected iron(III) porphyrin (Figure 2, **5**) is a high-spin complex (g = 6.393, 2.073) [24] that displays a Soret band at 408 nm (Figure 3). The corresponding water-free compound (referred to as **6**) exhibits a UV_{max} of 400 nm in agreement with CPO (398 nm) [25] which, according to X-ray analysis, is five coordinate [10]. It was shown that **5** reacts quantitatively with the hypochlorite **7** (Figure 2) to yield the adduct **8** (Figure 2), a high-spin complex (g = 6.090, 2.071) with a characteristic UV_{max} = 404 nm (Figure 4). The complex **8** (Figure 2) proved to be unreactive towards monochlorodimedone (**1**), but protonation produced the HOCl adduct (**9**), which then catalyzed the chlorination of **1** to give **2** (Figure 2). The complex **9** was characterized by MALDI-TOF mass spectroscopy (m/z = 1325 Da [M^+], ESR spectroscopy (g = 6.216, 2.071) and UV spectroscopy (λ_{max} = 388 nm, 416 nm; Figure 3). To determine whether the adducts **8** and **9** could be produced with the same reagents used by CPO, **5** was first treated with H_2O_2. Surprisingly, a single complex **10** (Figure 2) was detected in the UV spectrum at room temperature which, according to its λ_{max} (= 388 nm), is

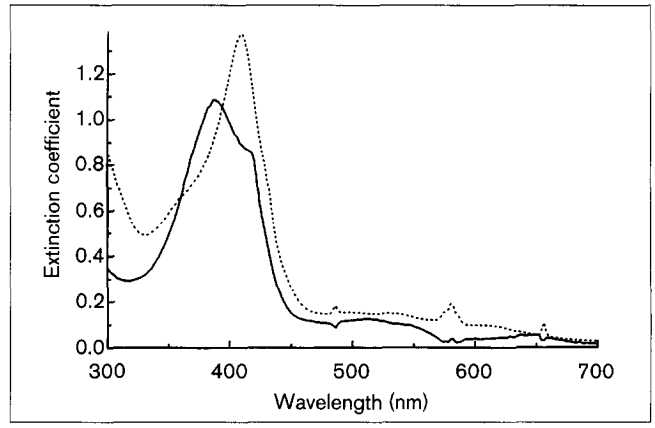
Figure 2

Chlorination of monochlorodimedone (**1**) catalyzed by the enzyme model **9**. **9** can be generated from the resting state model **5** via the intermediate **8**.



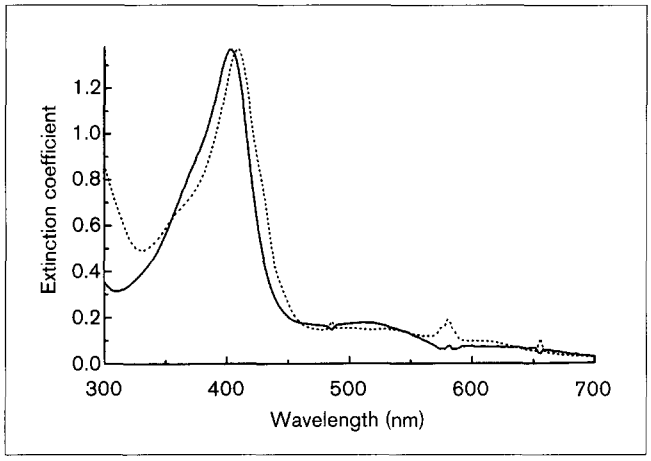
equivalent to compound I. On addition of Cl^- to **10** the adduct **8** was observed. After protonation, **8** gave the catalytically active HOCl adduct **9** (Figure 2). It has also been shown [24] that Lewis acids react with **8** to yield adducts displaying UV spectra corresponding to those of **9**. These compounds, which have the proton in **9** replaced by Lewis acids, chlorinate substrates with fourfold higher turnovers than **9**. Thus, the intermediates **8** and **9** can be generated via two distinct reaction pathways. These results indicate that iron(III) porphyrins with a thiolate ligand readily coordinate with ^-OCl and HOCl and mimic the catalytic behaviour of chloroperoxidase. It is important to note, however, that intermediates, like **8** and **9**, have never been observed in the enzymic reaction. We reasoned that these six-coordinate complexes may not have been detected because under common reaction conditions at pH < 3 intermediates corresponding to **8** and **9** are too short lived.

Figure 3



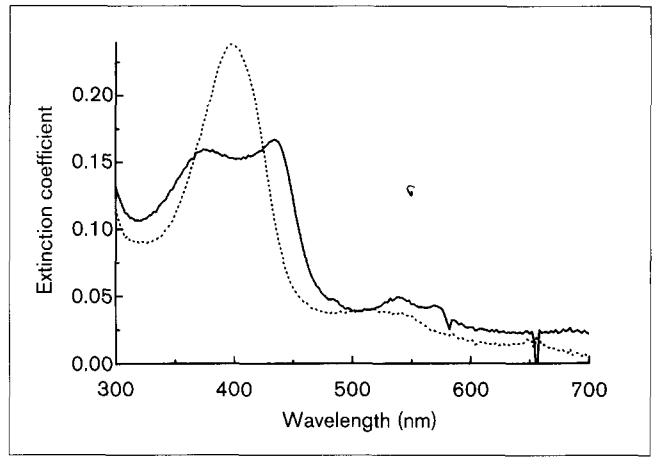
UV/visible spectra of the enzyme models **9** (HOCl–Fe(III)–S^-, unbroken line) and **5** (H_2O–Fe(III)–S^-; dotted line).

Figure 4



UV/visible spectra of the enzyme models **8** (ClO^-–Fe(III)–S^-, unbroken line) and **5** (H_2O–Fe(III)–S^-; dotted line).

Figure 5



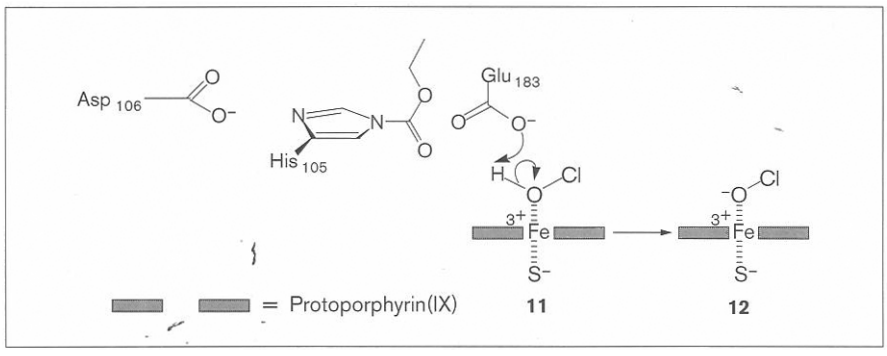
UV/visible spectra of the HOCl–CPO adduct **11** (unbroken line) and the resting state of CPO **4** (dotted line).

Thus, our first objective was to investigate the binding of HOCl and ^-OCl to commercially available CPO from *C. fumago* at different pH values. Screening the range pH3–9 at room temperature it became evident that at pH4.4 a new compound was formed. The Soret band at λ_{max} = 398 nm seen for the resting state disappeared and a split Soret band with maxima at 376 nm and 434 nm was observed (Figure 5) similar to the UV spectrum of **9**, the synthetic enzyme model. Therefore we conclude that under these conditions the HOCl-adduct of CPO (Figure 6, **11**) is produced. The splitting of the Soret band of **11** (Figure 5) and of **9** (Figure 3) confirms an earlier prediction based on molecular orbital calculations [26] that, due to the presence of the proximal thiolate ligand, heme-thiolate proteins should exhibit a split Soret band on addition of a sixth ligand. Until now this phenomenon has only been demonstrated for the oxygen complex (·O–O–Fe(III)–S^-) and the carbon monoxide complex (OC–Fe(II)–S^-) of cytochromes P450. For iron porphyrin model compounds, however, often only one of the two Soret bands is detectable.

In view of this result it seemed unlikely that any information on the existence of a ^-OCl–CPO adduct would be obtained using the intact enzyme. We therefore took advantage of the recently published X-ray crystal structure of CPO which identified Glu183 as part of a proton relay system including His105 and Asp106 [10]. Glu183 is located adjacent to the peroxide-binding site, suggesting that it is actively involved in peroxide cleavage (Figure 1). Because the covalent modification of His105 [27], using diethyl pyrocarbonate, inactivates CPO irreversibly and completely [28] it was anticipated that the ^-OCl–CPO adduct would be detectable by interrupting the proton supply. At pH6.0, His105 is the only residue of the proton shuttle system which remains protonated and therefore reacts with diethyl pyrocarbonate in dry ethanol. The reaction can be monitored by the increasing absorbance at λ = 246 nm [27].

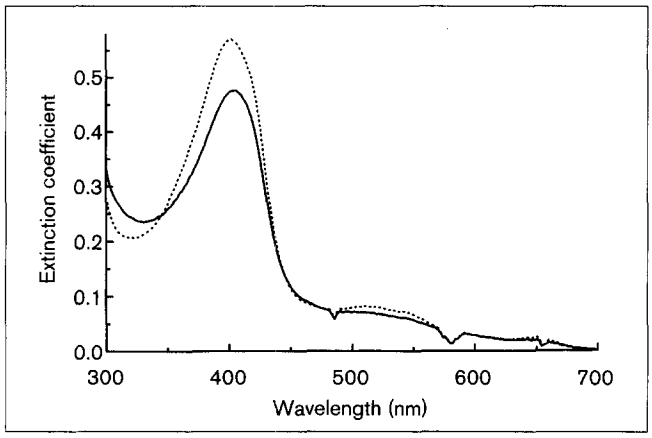
When CPO, containing the ethoxycarbonyl-protected His105, was treated with a NaOCl solution at pH6.0, a

Figure 6



Generation of the ClO^-–CPO intermediate **12** using a His105-modified CPO at pH=6.0.

Figure 7



UV/visible spectra of the $^{-}$OCl–CPO intermediate **12** (unbroken line) and the resting state of CPO **4** (dotted line).

significant change the UV spectrum was detected: the value of λ_{max} (398 nm) seen for the resting state of CPO changed to λ_{max} = 406 nm (Figure 7). When compared with the enzyme model **8** (λ_{max} = 404 nm) this can be interpreted in terms of the formation of the $^{-}$OCl–CPO adduct **12**. Given the experimental conditions, namely pH = 6.0, and the pK_a values for glutamate (pK_a = 4.27) and HOCl (pK_a = 7.49), we believe that HOCl first coordinates to iron and is then sufficiently acidic to protonate the adjacent Glu183 (Figure 6). It is important to note that on adjusting the acidity to pH = 3.8 the absorbance of the $^{-}$OCl adduct remained unchanged and the UV maxima of the HOCl–CPO adduct were not detectable, indicating that the proton delivery was completely blocked.

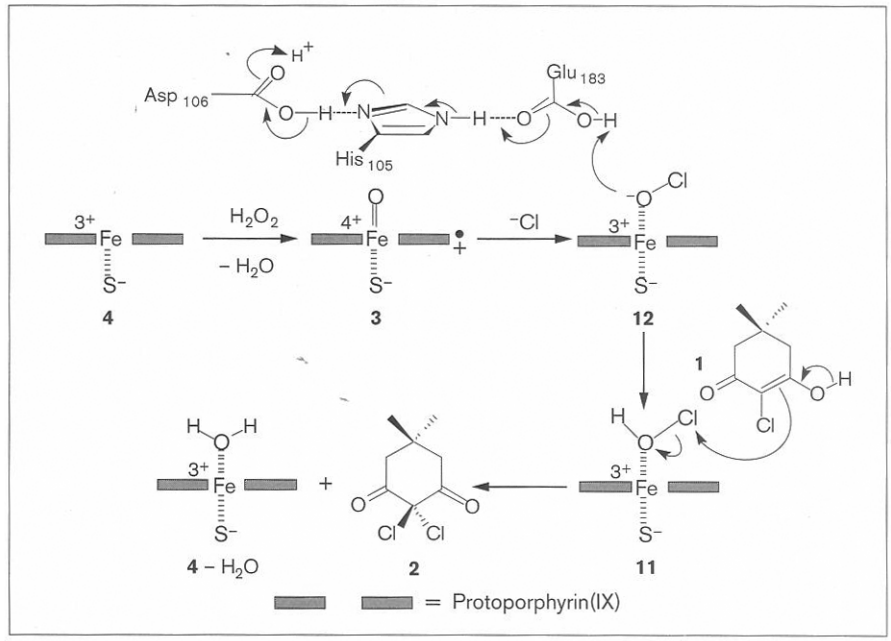
These experiments demonstrate that the $^{-}$OCl adduct and the HOCl adduct of the iron(III) protoporphyrin(IX) complex of CPO can be generated at pH = 6 and pH = 4.4, respectively. Both pH values are notably different from the pH at which CPO performs the chlorination using H_2O_2 and Cl^{-} (pH < 3). It seems reasonable, however, that neither intermediate is observable at low pH under conditions when the proton delivery system is fully operative and, as we know from enzyme model studies, the final Cl^{+} donor is very reactive. We therefore propose a reaction mechanism for CPO (Figure 8) involving intermediates **11** and **12** that is consistent with our observations and is supported by recent kinetic studies [29]. After the cleavage of H_2O_2, (see Figures 1,8) the oxo iron intermediate **3** is generated. Addition of Cl^{-} to **3** (Figure 8) leads to the $^{-}$OCl–CPO adduct **12** which is immediately protonated by the proton relay system to yield the HOCl–CPO complex **11**. This is a catalytically active Cl^{+} donor as is its equivalent (**9**) in the synthetic enzyme model.

Significance

The spectroscopic identification of reactive intermediates in the catalytic cycle of chloroperoxidase, reported here for the first time, provides important clues to the enzymic reaction mechanism of this heme-thiolate protein. This study also highlights the usefulness of synthetic model compounds that are closely related to an enzyme's active site. Such enzyme models could become increasingly important as guides to understanding enzymic catalysis.

Figure 8

Proposed reaction mechanism of CPO.



Materials and methods

Materials

All reagents purchased were of suitable grade and purity for immediate use. CPO (1350 U mg^{-1}; 2.8 mg ml^{-1}) and $NaOCl_{aq}$ (5% chlorine) were purchased from Sigma Chemicals. Diethyl pyrocarbonate, dry ethanol (<0.01% H_2O) and all other chemicals were purchased from Fluka Chemicals. CPO was stored at 4°C and diethyl pyrocarbonate was stored at −20°C.

UV spectroscopy

Absorption measurements were performed using a Hewlett Packard 8452A diode array spectrophotometer. Spectra were collected at room temperature using Hellma quartz cuvettes (1 cm), and were recorded with a Hewlett Packard UV-Visible Chemstation (Rev. A.02.04).

Spectroscopy of CPO

The concentration of CPO was determined by the absorbance at 398 nm (Soret band) using an extinction coefficient of 85000 M^{-1} cm^{-1} [30]. 2.0 nmol CPO were dissolved in 700 µl potassium or sodium phosphate buffer at different pH values (2.7, 4.4, 6.9 and 9.2). After addition of a 500-fold excess of $NaOCl_{aq}$ at room temperature the absorbance was immediately recorded. A significant change of the Soret band was only observed at pH 4.4. **11**: UV/visible (H_2O): λ_{max} (%) = 376 nm (100), 434 (100), 538 (30), 570 (26).

Modification of CPO with diethyl pyrocarbonate

Solutions of diethyl pyrocarbonate in dry ethanol (<5% v/v) were prepared for each experiment just before use, and stored at −20°C for a few hours only. The concentration of diethyl pyrocarbonate solutions were determined by addition of small aliquots (1–5 µl) to 2.4 ml of 10 mM 2-methyl imidazole. The concentration was calculated by linear regression and by the absorbance at 230 nm using an extinction coefficient of 3000 M^{-1} cm^{-1} [27].

The modification of CPO has been shown to be specific for histidine residues between pH 5.5 and pH 7.5 [27]. For this reason a 100 mM potassium phosphate buffer at pH 6.0 was chosen for the derivatization. The concentration of CPO was determined by the absorbance at 398 nm using an extinction coefficient of 85000 M^{-1} cm^{-1} [30]. According to the literature [28] a 500-fold excess of diethyl pyrocarbonate was added to 5.4 nmol CPO in 700 µl buffer at room temperature to acylate only one histidine residue. The formation of the *N*-ethoxycarbonyl histidine was controlled by recording the increase of the absorption at 246 nm, and the stoichiometry was determined using an extinction coefficient of 3200 M^{-1} cm^{-1} [27]. The reaction was started by the addition of diethyl pyrocarbonate and terminated when the absorbance at 246 nm reached its maximum value. A ratio of 1.2:1 was calculated for *N*-ethoxycarbonyl histidine over CPO.

Spectroscopy of modified CPO

After addition of a 500-fold excess of $NaOCl_{aq}$ at room temperature to a solution of 5.4 nmol of the modified CPO in 700 µl potassium phosphate buffer at pH 6.0 the UV spectrum was recorded immediately. **12**: UV/visible (H_2O): λ_{max} (%) = 406 nm (100), 576 (20), 656 (17). Another sample of 5.4 nmol CPO in 700 µl potassium phosphate buffer at pH 6.0 was acidified with glacial acetic acid to pH 3.8. As described above, an excess of $NaOCl_{aq}$ was added to the solution and the UV spectrum measured immediately. **12**: UV/visible (H_2O): λ_{max} (%) = 408 nm (100), 576 (21), 656 (19).

Acknowledgements

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