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The ferric-hydroperoxo complex of chloroperoxidase

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Abstract

The hydroperoxo-ferric complex, or Compound 0 (Cpd 0), is an unstable transient intermediate common for oxygen activating heme enzymes such as the cytochromes P450, nitric oxide synthases, and heme oxygenases, as well as the peroxidases and catalases which utilize hydrogen peroxide as a source of oxygen and reducing equivalents. Detailed understanding of the mechanism of oxygen activation and formation of the higher valent catalytically active intermediates in heme enzyme catalysis requires the structural and spectroscopic characterization of this immediate precursor, Cpd 0. Using the method of cryoradiolytic reduction of the oxy-ferrous heme complex, we have prepared and characterized hydroperoxo-ferric complex in chloroperoxidase (CPO) and compared this to the same intermediate generated in cytochrome P450 CYP101. Optical absorption spectrum of Cpd 0 in CPO has a Soret band at 449 nm and poorly resolved α, β bands at 576 and 546 nm.

Keywords: Chloroperoxidase; Cytochrome P450; Peroxo-ferric intermediate; Cryogenic spectroscopy; Radiolytic reduction; Heme enzyme

The hydroperoxo-ferric intermediate, or ''Compound 0'', is a common intermediate in heme enzyme catalysis [1]. It appears as a distinct transient species on the path to the higher valent ferryl-oxo states in the enzymatic cycles of the cytochromes P450, nitric oxide synthases, and heme oxygenases, all of which reduce dioxygen, as well as in heme peroxidases and catalases in their reactions with hydrogen peroxide (Scheme 1). Compound 0 (Cpd 0) is intrinsically unstable and usually decomposes at ambient conditions faster than it forms, and hence is extremely difficult to observe and characterize in the native reactions [2,3]. However, Cpd 0 can be obtained via cryogenic radiolytic reduction of the one-electron precursor oxy-ferrous complex in the frozen solutions or crystals [4–6]. Using this method, hydroperoxo-ferric complexes were characterized by electron paramagnetic resonance (EPR) [7–17], optical absorption [15,18–20], and Raman spectroscopy [20,21] in cytochrome P450 [12,18,21], myoglobin and hemoglobin [7–9,20], heme oxygenase [14,17,19], and nitric oxide synthase [13].

The various features of hydroperoxo-ferric intermediates in the heme enzymes and model compounds have been reviewed [1,22–25]. Specifically, in all heme enzymes the EPR spectra of the invariably ferric low-spin peroxo-/hydroperoxo- complexes show typical narrow span of g values regardless of the axial ligand [23]. The optical absorption spectra are in turn sensitive to the nature of the proximal ligand *trans* to coordinated hydroperoxide. In myoglobin, heme oxygenase, and horseradish peroxidase, which all have His-ligated heme iron, the Soret band is red-shifted by 3–8 nm as a result of the one-electron reduction of the oxy-ferrous precursor, while in cytochromes P450 and CPO this shift is larger, 20–22 nm [1]. Based on these spectral signatures, EPR and UV–vis absorption spectroscopy are becoming the methods of choice for the assignment of the transient intermediates

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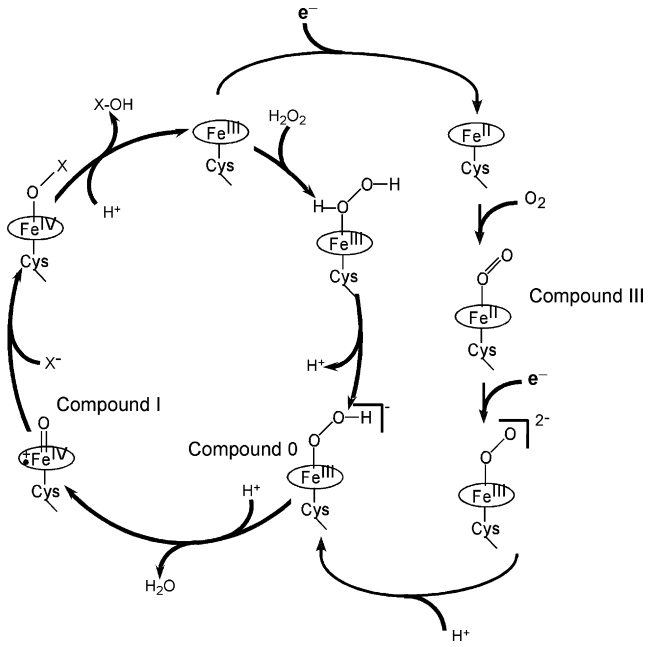

Scheme 1. The reaction cycles of chloroperoxidase (shown in the left cycle with the pathway from the resting state to Cpd0 utilizing hydrogen peroxide as a source of electrons and oxygen) and cytochrome P450 (the right pathway, utilizing dioxygen and two external electrons from redox partners). Compound 0 and Compound I are common intermediates for peroxidases and cytochromes P450.

observed in the reactions of hydrogen peroxide with the heme enzymes [2,3,25–29].

Recently the first high resolution X-ray structure of Cpd 0 in chloroperoxidase was published [6]. In these experiments, Cpd 0 was obtained via *in situ* radiolytic reduction of Compound III (Cpd III), the ferrous oxygenated state [30,31], which was prepared by soaking crystals of the ferric CPO in peracetic acid. The redox state of the enzyme during X-ray irradiation and data collection was verified using absorption spectroscopy, and the assignment of Cpd 0 was based on the pronounced red shift of Soret band as compared to the spectrum of oxy-ferrous complex (Cpd III) in CPO single crystals before irradiation [6]. However, the anisotropic optical properties of protein crystals and the variability of the spectra with respect to the position of the sample make it necessary to compare the results obtained by single crystal microspectroscopy to the spectra measured using isotropic frozen solutions. For instance, the position of the Soret band of the Cpd III in CPO was identified between 430 and 445 nm in the crystal [6], while in solution it was reported at 427 nm [30,31]. The red shift and apparently low amplitudes of Soret bands in the single crystal spectra [6] may be attributed to the high background and/or scattering in single crystal cryogenic microspectroscopy, complicating the precise assignment of the Soret maximum. In order to provide an absolute spectral assignment of oxy-ferrous and hydroperoxo-ferric intermediates of CPO, we document in this communication the high resolution optical spectra of these unstable intermediates measured at 77 K in comparison with the spectra of the same complexes in cytochrome P450.

Methods

Chloroperoxidase isolated from the fungi *Caldariomyces fumago* (CPO) was prepared in the laboratory of Dr. L. Hager as described [32]. Samples for cryogenic spectroscopy were prepared in 0.1 M phosphate buffer, pH 6.0, containing 65% v/v glycerol. The substrate thioanisole ($C_6H_5SCH_3$) was added as 0.5 M stock solutions in ethanol. Ferrous CPO was prepared by addition of minimal amounts of dry sodium dithionite and subsequent anaerobic buffer exchange using small G25 Sepharose (Pharmacia) to remove unreacted reductant and unwanted by-products. Oxy-ferrous CPO was obtained by gentle bubbling of oxygen gas into the solution of reduced enzyme at 255 K followed by stirring as described [15,18]. The samples were placed into the pre-cooled optical cryostat (details of construction to be found in reference [4]), and the spectra were acquired while cooling to 80 K using liquid nitrogen.

Radiolytic reduction was accomplished by γ-irradiation of the samples immersed in liquid nitrogen using a ^{60}Co-source for 210 min at the dose rate 16.8 krad/min with a total dose of 3.5 Mrad. The yield of Cpd 0 in CPO was estimated based on the optical absorption of the oxy-ferrous CPO before and after irradiation [4,33].

Results and discussion

The observed temperature dependent spin equilibrium in ferric CPO at pH 6.0 in the absence and in the presence of the substrate thioanisole is shown in Fig. 1 as the sample of the ferrous-oxy complex is cooled following oxygenation. In agreement with earlier observations [34,35] at pH 6.0, CPO undergoes a transition from predominantly high-spin state at the ambient conditions to the low-spin state at 77 K, Fig. 1. This spin shift is caused by coordination of the oxygen atom from the water molecule at the sixth position of the heme iron, resolved in the X-ray structure of the substrate free CPO [36]. The presence of thioanisole did not change the apparent spin state of CPO as monitored by optical spectroscopy. At room temperature, a broad Soret maximum is observed at 404 nm, while at below 170 K the spectrum in the Soret region is represented by the typically sharp low-spin band at 423 nm with a broad band at 355 nm. In the visible region, the temperature decrease results in the gradual replacement of the high spin bands at 515 nm and 650 nm by the low-spin spectra with two well-resolved peaks at 541 and 582 nm and several weak broad bands at 650–750 nm. Following reduction at the ambient conditions, the main maximum of the Soret band is observed at 409 nm with the shoulders at 421 nm and 446 nm.

Spectra of the oxy-ferrous CPO are illustrated in Fig. 2. As reported earlier [31], the Soret band clearly reveals features similar to those observed for the oxy-complexes of all thiolate-ligated heme protein, including the distinctive so-called "split Soret" [37–41]. In the low temperature spectra of the oxy-complex of CPO, the main Soret band is observed at 428 nm, and the broad secondary band at 354 nm. In the visible region, the α and β bands are well resolved at $T < 170$ K with the maxima at 553 nm and 587 nm.