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Observation of the Fe^{IV}=O stretching Raman band for a thiolate-ligated heme protein

Compound I of chloroperoxidase

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The Fe^{IV}=O stretching vibration has never been identified for a cysteine-coordinated heme enzyme. In this study, resonance Raman and visible absorption spectra were observed simultaneously for transient species in the catalytic reaction of chloroperoxidase with hydrogen peroxide by using our original apparatus for mixed-flow and Raman/absorption simultaneous measurements. For the first intermediate, the Fe^{IV}=O stretching Raman band was observed at 790 cm^{-1}, which shifted to 756 cm^{-1} with the ^{18}O derivative, but the ν_4 band was too weak to be identified. This suggested the formation of an oxoferryl porphyrin π cation radical. The second intermediate gave an intense ν_4 band at 1,372 cm^{-1} but no oxygen isotope-sensitive Raman band, suggesting oxygen exchange with bulk water.

Chloroperoxidase; Compound I; Compound II; Ferryl-oxo stretching mode; Resonance Raman; Thiolate-ligated heme

1. INTRODUCTION

Chloroperoxidase (CPO), a thiolate-ligated heme protein isolated from *Caldariomyces fumago*, catalyzes the hydrogen peroxide dependent formation of carbon–halogen bonds in the presence of halogen anions (Cl^-, Br^-, or I^-) and an organic substrate like β-diketone [1]. One of the characteristic structural features on the catalytic site of CPO is the coordination of cysteine sulfur anion as the fith ligand of the heme iron [2], which is seen for cytochromes P-450 (P-450) in common [3,4]. Despite of similarities between CPO and P-450 in their physical properties such as the Fe^{III}–S bond lengths [5] and the Fe^{III}–S stretching frequencies [6], their reactivities are quite different; P-450 serves as a monooxygenase but CPO works as a peroxidase. It is well known that the first intermediate in the catalytic reaction of a peroxidase with hydrogen peroxide, called compound I, contains two extra oxidative equivalents compared with the ferric enzyme (see [7] for a review). One of the extra oxidative equivalents is contained in the Fe^{IV}=O bond and the other is contained in the porphyrin macrocycle or the protein moiety as a cation radical. When an electron is donated by a substrate to compound I, it is converted to compound II with the Fe^{IV}=O heme.

So far the Fe^{IV}=O heme has never been identified for an S^--coordinated heme enzyme, although its presence in P-450 catalysis has been deduced from chemistry of model compounds [8]. The Fe^{IV}=O stretching (ν_{Fe-O}) Raman band of peroxidase intermediates are expected to serve as a sensitive indicator of the environments around the bound oxygen [9]. Previously, we successfully applied the mixed-flow transient Raman apparatus [10] and a novel device for Raman/absorption simultaneous measurement system [11] to observe the 00 stretching Raman band of oxygenated P-450 under catalytic conditions [12], but the ν_{Fe-O} band was not observable for successive intermediates. In order to establish the Fe=O stretching frequency of an S^--coordinated heme enzyme, we studied the reaction of CPO with hydrogen peroxide and, for its compound I species, observed it at 790 cm^{-1}.

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2. MATERIALS AND METHODS

CPO was isolated from culture filtrates of *C. fumago* and purified according to Morris and Hager [1]. To obtain the spectra of compound I, 60 μM CPO solution in 100 mM phosphate buffer, pH 3.5 was mixed with the same volume of 1 mM hydrogen peroxide solution by using the mixed flow apparatus. While the Raman spectrum was measured for the mixed and flowing solutions, the visible absorption spectrum of the scattering volume was monitored between 500 and 800 nm with the device for Raman/absorption simultaneous measurements. The Raman spectrum of compound II was obtained in the same way except that 3 mM ascorbic acid, an electron donor to compound I, was contained in the hydrogen peroxide solution. $H_2{}^{18}O_2$ was synthesized previously [13]. Raman scattering was excited by the 363.8 nm line of an Ar iron laser (Spectra Physics, 2045) and detected by an intensified

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photodiode array (PAR 1421-HQ) attached to a double monochromator (Spex 1404).

3. RESULTS AND DISCUSSION

Fig. 1 depicts visible absorption spectra of the resting CPO (A), compound I (B) and compound II (C) observed with the present method. These are in agreement with the reported spectra of each species [14, 15]. Therefore, compound I is in fact generated in the flowing solution and is not photoreduced by the Raman probe light under the present conditions. Fig. 2 shows the Raman spectra observed simultaneously with the absorption spectra shown in Fig. 1. Spectra B and C were obtained for compound I derived from $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$, respectively, and trace D exhibits their difference [D = 2×(B−C)]. This indicates that the band at 790 cm^{-1} of spectrum B is shifted to 756 cm^{-1} in spectrum C. The observed frequency shift (34 cm^{-1}) is in excellent agreement with the theoretical value (35 cm^{-1}) expected for an isolated Fe=O diatomic oscillator, implying little coupling with other vibrational modes. Since this band exhibited no frequency shift in D_2O, it cannot be assigned to the O–O stretching mode (ν_{OO}) of the Fe^{III}–O–O–H structure which was recently identified at 787 cm^{-1} for a reaction intermediate (compound B) of cytochrome *c* oxidase [16]. Consequently, the 790 cm^{-1} band of spectrum B is assigned to ν_{FeO} of CPO compound I.

Previous studies on model porphyrin systems indicate that the ν_4 band exhibits a significant downshift and weakening upon formation of π cation radical [17–20]. In contrast, compound ES of cytochrome *c* peroxidase, which has the Fe^{IV}=O heme and protein cation radical, gives an intense ν_4 band at 1378 cm^{-1} [21]. The ν_4 band of resting CPO appears at 1372 cm^{-1} as shown by spectrum A, but the corresponding band of compound I is too weak to be identified. This feature is compatible with the assignment of the CPO compound I to the porphyrin π cation radical, although spectrum B is greatly different from the spectra of horseradish peroxidase (HRP) compound I [22–24].

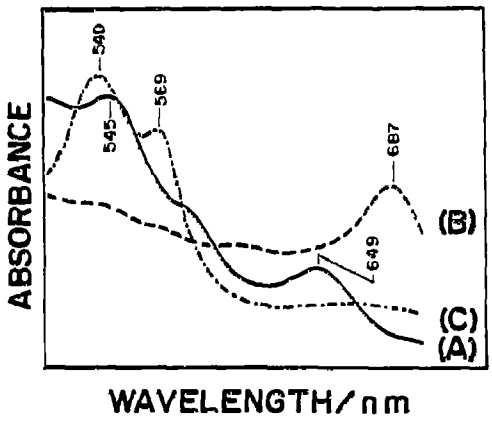

Fig. 1. Visible absorption spectra of the resting CPO (A), compound I (B), and compound II (C) observed with the device for Raman/absorption simultaneous measurements.

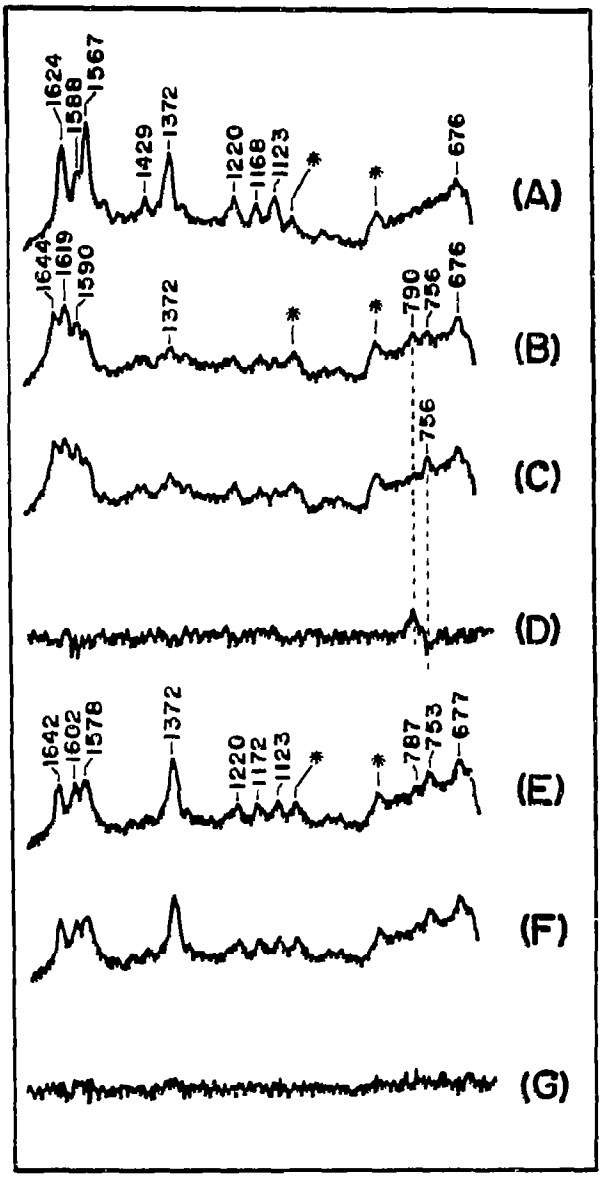

Fig. 2. Resonance Raman spectra of CPO excited at 363.8 nm. (A) Resting CPO, (B) compound I derived from $H_2{}^{16}O_2$, (C) compound I derived from $H_2{}^{18}O_2$, (D) difference spectrum, 2×(spectrum B − spectrum C), (E) compound II derived from $H_2{}^{16}O_2$, (F) compound II derived from $H_2{}^{18}O_2$, (G) difference spectrum, 4×(spectrum E − spectrum F). Spectra A, B and E were observed simultaneously with the absorption spectra A, B and C of Fig. 1, respectively. Asterisks denote the Raman bands of buffer solution.

Spectra E and F in Fig. 2 were observed for compound II derived from $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$, respectively, and trace G depicts the expanded difference spectrum [G = 4×(E-F)]. We failed to find an oxygen-isotope sensitive band in the spectrum of compound II. Since

spectrum E in the 1570–1650 cm^{-1} region is distinct from spectrum A, occurrence of photoreduction during the Raman measurements is unlikely. It is highly likely that the Fe^{IV}=O oxygen is exchanged with bulk water as noted for HRP compound II at neutral pH [25,26].

In order to discuss the trans ligand effect on the Fe^{IV}=O bond, one should compare the $\nu_{Fe=O}$ frequency of CPO compound I with those of other compounds I, but they are not available; the Raman spectra of HRP compound I are still controversial [22–24]. On the other hand, the $\nu_{Fe=O}$ frequency of a model Fe^{IV}=O porphyrin was shown to be scarcely altered by formation of π cation radical [20,27]. This may allow us to compare the $\nu_{Fe=O}$ frequency of CPO compound I with those of compound II of other peroxidases. The $\nu_{Fe=O}$ frequency of CPO compound I (790 cm^{-1}) is comparable with that of HRP-C at pH 11.2 (787 cm^{-1}) [13,25,28], and is appreciably higher than those of other histidine-coordinated peroxidases including HRP-C at pH 7.0 (774 cm^{-1}) [25,28], HRP-A (779 cm^{-1}) [26,29], myeloperoxidase (782 cm^{-1}) [30], and cytochrome *c* peroxidase (767 cm^{-1}) [21], but is lower than that of myoglobin (+H_2O_2) (797 cm^{-1}) [31]. Therefore, the $\nu_{Fe=O}$ frequency depends on the environments around the oxygen atom, and the effect from coordination of the S^- ligand upon the Fe^{IV}=O bond at its transposition seems to be small. The specific reactivity of CPO must be determined by amino acid residues in the distal side.

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