Oxygen exchange between the Fe(IV)=O heme and bulk water for the A_2 isozyme of horseradish peroxidase

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Resonance Raman spectra were observed for compound II of horseradish peroxidase A_2, and the Fe(IV)=O stretching Raman line was identified at 775 cm^{-1}. This Raman line shifted to 741 cm^{-1} upon a change of solvent from $H_2{}^{16}O$ to $H_2{}^{18}O$, indicating occurrence of the oxygen exchange between the Fe(IV)=O heme and bulk water. The oxygen exchange took place only at the acidic side of the heme-linked ionization with $pK_a = 6.9$.

Resonance Raman spectroscopy *Peroxidase* *Heme protein* *Oxo-ferryl porphyrin* *Iron porphyrin*

1. INTRODUCTION

Observation of the Fe(IV)=O stretching Raman line for compound II of horseradish peroxidase (HRP) provided conclusive evidence for the oxo-ferryl structure of the heme [1,2] and furthermore revealed the presence of hydrogen bonding between the oxygen atom of the Fe(IV)=O heme and an adjacent amino acid residue [3,4]. The hydrogen atom involved in the hydrogen bond was demonstrated to be the dissociable proton in the heme-linked ionization, and thus with Raman spectroscopy, we stepped forward to understanding of the structural implication of the heme-linked ionization [5]. However, there was still controversy to be solved between two Raman studies; Hashimoto et al. [4] pointed out the oxygen exchange between the Fe(IV)=O heme and bulk water for HRP isozyme C (HRP-C) whereas the Raman spectrum of HRP isozyme A_2 (HRP-A_2) reported by Sitter et al. [3] implied no oxygen exchange. In modeling the catalytic mechanism by this enzyme, it is essentially important to determine whether the oxygen exchange is a characteristic property of a peroxidase and has relevance to the enzyme reaction. Accordingly, in this study, we investigated resonance Raman (RR) spectra of compound II of HRP-A_2 and report definite evidence for the oxygen exchange.

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Abbreviations: RR, resonance Raman; HRP, horseradish peroxidase; HRP-A_2, isozyme A_2 of horseradish peroxidase; HRP-C, isozyme C of horseradish peroxidase

2. MATERIALS AND METHODS

HRP-A prepared as in [6] was split into A_1, A_2 and A_3 components according to Shannon et al. [7]. The split A_2 component with RZ = 4.1 was concentrated with $(NH_4)_2SO_4$ and centrifugation, desalted by dialysis against distilled water at 4°C, and then dissolved in phosphate or acetate buffer according to neutral or acidic pH, respectively. Compound II was obtained by addition of equimolar hydrogen peroxide to an equimolar mixture of the resting enzyme and ferrocyanide. $H_2{}^{18}O_2$ was synthesized as described [4]. $H_2{}^{18}O$

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was purchased from Amersham International. Raman spectra were excited by the 406.7 nm line of a Kr^+ ion laser (Spectra Physics, 165) and detected with a Reticon diode array detector (PAR 1420) attached to a Spex double monochromator. Since compound II is photolabile, the sample in a spinning cell (1800 rpm, diameter 2 cm) was excited with very weak laser power (5 mW) and the accumulation time was made short (3 min).

3. RESULTS AND DISCUSSION

Fig.1 shows the RR spectra of resting HRP-A_2 (a) and compound II (b,c) at pH 8.4. The Raman line of compound II at 787 cm^{-1} (b), which was absent in (a), was shifted to 753 cm^{-1} (c) upon replacement of $H_2{}^{16}O_2$ with $H_2{}^{18}O_2$ and was therefore assigned to the Fe(IV)=O stretching mode, in agreement with the result of Sitter et al. [3]. Fig.2 shows the results at pH 5.3. Compound II derived with $H_2{}^{16}O_2$ in $H_2{}^{16}O$ (b) gave a new Raman line at 775 cm^{-1}, which was absent in the native enzyme (a). In contrast with the results at pH 8.4, the 775 cm^{-1} line did not exhibit an isotopic frequency shift upon replacement of

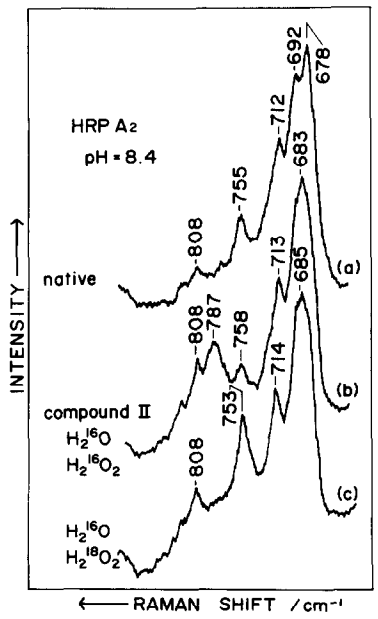

Fig.1. RR spectra of resting HRP-A_2 and compound II at pH 8.4; (a) resting enzyme, (b) compound II derived from $H_2{}^{16}O_2$, (c) compound II derived from $H_2{}^{18}O_2$.

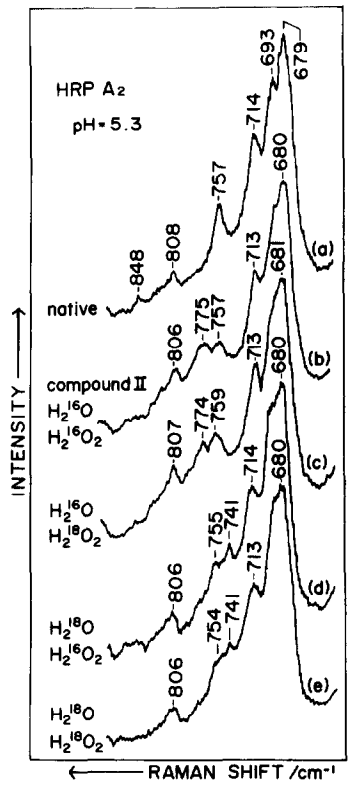

Fig.2. RR spectra of resting HRP-A_2 and compound II at pH 5.3; (a) resting enzyme, (b) compound II derived from $H_2{}^{16}O_2$ in $H_2{}^{16}O$, (c) from $H_2{}^{18}O_2$ in $H_2{}^{16}O$, (d) from $H_2{}^{16}O_2$ in $H_2{}^{18}O$, (e) from $H_2{}^{18}O_2$ in $H_2{}^{18}O$.

$H_2{}^{16}O_2$ with $H_2{}^{18}O_2$ (c). However, this Raman line disappeared and a new line appeared at 741 cm^{-1} in $H_2{}^{18}O$ irrespective of using $H_2{}^{16}O_2$ (d) or $H_2{}^{18}O_2$ (e). The extent of the frequency change agrees closely with that (35 cm^{-1}) expected for replacement of ^{16}O with ^{18}O of the Fe=O two-body harmonic oscillator. Therefore, the 775 and 741 cm^{-1} lines are assigned to the Fe(IV)=^{16}O and Fe(IV)=^{18}O stretching modes, respectively. Fig.3 shows the RR spectra of compound II in ^2H_2O at p^2H 5.3. The 775 cm^{-1} line is more intense in ^2H_2O than in ^1H_2O as previously noted for HRP-C [4]. This line showed no frequency shift when $H_2{}^{18}O_2$ was used instead of $H_2{}^{16}O_2$ to derive compound II. These results evidently indicate that the heme-bound oxygen atom is exchanged with that of bulk water at pH 5.3.

The Fe(IV)=O stretching Raman line appears at 787 cm^{-1} at pH 8.3 (fig.1) but at 775 cm^{-1} at pH

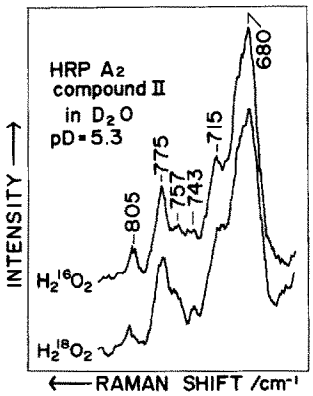

Fig.3. RR spectra of compound II of HRP-A_2 in ^2H_2O at p^{2}H 5.3 derived from $H_2{}^{16}O_2$ (upper) and $H_2{}^{18}O_2$ (lower).

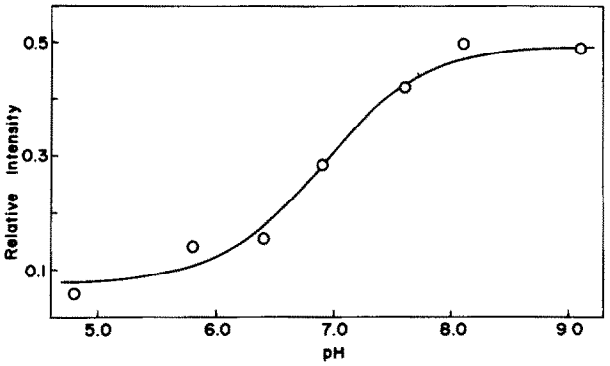

Fig.4. pH dependence of the 787 cm^{-1} line of compound II. Relative peak intensity of the 787 cm^{-1} line of compound II to the 983 cm^{-1} line of the sulfate ion (I_{787}/I_{983}) is plotted vs pH (○). (——) Theoretical curve expected for deprotonation of one proton with pK_a 6.9.

5.3 (fig.2). The lower frequency at acidic pH suggests the presence of hydrogen bonding to the oxygen atom. The relative intensity of the 787 cm^{-1} line to the 983 cm^{-1} line of SO_4^{2-}, which was added to the sample solution as an internal intensity standard, is plotted vs pH in fig.4, where the solid line represents the theoretical curve expected for deprotonation of one proton with pK_a 6.9. This implies that the proton involved in the hydrogen bond dissociates from the residue with pK_a 6.9.

A kinetic study on HRP-A_2 [8] indicated that the rate of the reaction from compound II to the native state was slowed down at the alkaline side of the heme-linked ionization with pK_a 6.9. Therefore, it is now confirmed for HRP-A_2 that the hydrogen-bonded proton plays a key role in converting the oxygen atom of the Fe(IV)=O heme to H_2O [3,4] and thus in the enzymic activity. It is stressed here that the pH dependence of this enzymic activity is parallel with that of the oxygen exchange. When the rate of the oxygen exchange is compared between HRP-A_2 at pH 5.3 and HRP-C at pH 7.0, it seems to be faster with HRP-A_2 than with HRP-C, because the Fe(IV)=^{18}O stretching band is missing in the lower spectrum of fig.3 while it was seen with nearly half the full intensity for HRP-C under the same experimental conditions [4]. The larger rate for HRP-A_2 than for HRP-C is compatible with its lower pK_a value when the dissociation of the proton is the rate-limiting step for the oxygen exchange. Consequently, it can be concluded that the oxygen exchange takes place for HRP-A_2 and seems to be a characteristic feature of the HRP catalysis.

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