

# Oxygen exchange between the Fe(IV)=O heme and bulk water for the A<sub>2</sub> isozyme of horseradish peroxidase

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Resonance Raman spectra were observed for compound II of horseradish peroxidase A<sub>2</sub>, and the Fe(IV)=O stretching Raman line was identified at 775 cm<sup>-1</sup>. This Raman line shifted to 741 cm<sup>-1</sup> upon a change of solvent from H<sub>2</sub><sup>16</sup>O to H<sub>2</sub><sup>18</sup>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<sub>a</sub> = 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<sub>2</sub> (HRP-A<sub>2</sub>) 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<sub>2</sub> and report definite evidence for the oxygen exchange.

## 2. MATERIALS AND METHODS

HRP-A prepared as in [6] was split into A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub> components according to Shannon et al. [7]. The split A<sub>2</sub> component with RZ = 4.1 was concentrated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub><sup>18</sup>O<sub>2</sub> was synthesized as described [4]. H<sub>2</sub><sup>18</sup>O

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*Abbreviations:* RR, resonance Raman; HRP, horseradish peroxidase; HRP-A<sub>2</sub>, isozyme A<sub>2</sub> of horseradish peroxidase; HRP-C, isozyme C of horseradish peroxidase



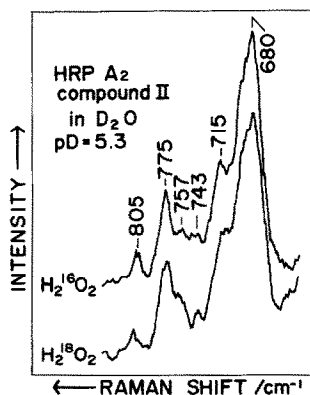


Fig.3. RR spectra of compound II of HRP-A<sub>2</sub> in <sup>2</sup>H<sub>2</sub>O at p<sup>2</sup>H 5.3 derived from H<sub>2</sub><sup>16</sup>O<sub>2</sub> (upper) and H<sub>2</sub><sup>18</sup>O<sub>2</sub> (lower).

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<sup>-1</sup> line to the 983 cm<sup>-1</sup> line of SO<sub>4</sub><sup>2-</sup>, 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<sub>a</sub> 6.9. This implies that the proton involved in the hydrogen bond dissociates from the residue with pK<sub>a</sub> 6.9.

A kinetic study on HRP-A<sub>2</sub> [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<sub>a</sub> 6.9. Therefore, it is now confirmed for HRP-A<sub>2</sub> that the hydrogen-bonded proton plays a key role in converting the oxygen atom of the Fe(IV)=O heme to H<sub>2</sub>O [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<sub>2</sub> at pH 5.3 and HRP-C at pH 7.0, it seems to be faster with HRP-A<sub>2</sub> than with HRP-C, because the Fe(IV)=<sup>18</sup>O stretching band is missing in the lower spectrum of fig.3 while it was seen with nearly half the full intensity for

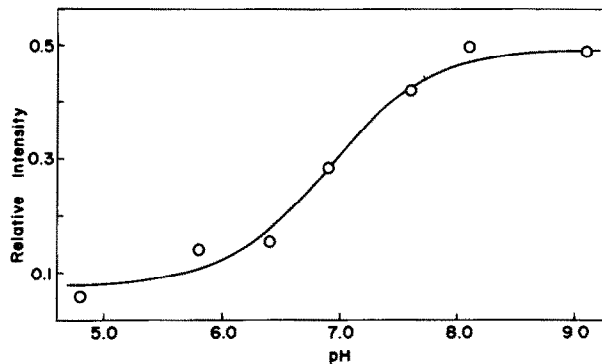


Fig.4. pH dependence of the 787 cm<sup>-1</sup> line of compound II. Relative peak intensity of the 787 cm<sup>-1</sup> line of compound II to the 983 cm<sup>-1</sup> line of the sulfate ion (*I*<sub>787</sub>/*I*<sub>983</sub>) is plotted vs pH (O). (—) Theoretical curve expected for deprotonation of one proton with pK<sub>a</sub> 6.9.

HRP-C under the same experimental conditions [4]. The larger rate for HRP-A<sub>2</sub> than for HRP-C is compatible with its lower pK<sub>a</sub> 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<sub>2</sub> and seems to be a characteristic feature of the HRP catalysis.

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