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Resonance Raman characterization of hog thyroid peroxidase

An SERRS study

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Resonance Raman (RR) spectra of hog thyroid peroxidase (TPO) were observed for the first time and compared with those of lactoperoxidase (LPO) and horseradish peroxidase (HRP). Since TPO purified by monoclonal antibody-assisted immunoaffinity chromatography was strongly fluorescent, the surface enhancement technique using Ag colloid adsorption was used for the oxidized form, but ordinary RR spectra could be obtained for the reduced form. The RR spectra of TPO were distinct from those of HRP in both the oxidized and reduced states and indicated the presence of six-coordinated iron-protoporphyrin.

Peroxidase; Resonance Raman spectroscopy; SERRS; (Thyroid)

1. INTRODUCTION

Thyroid peroxidase [(TPO) donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7] is a heme-containing membrane protein [1] that catalyzes the iodination of tyrosine residues in thyroid hormone biosynthesis [2]. Although it is a matter of concern whether the spectral properties of TPO resemble those of horseradish peroxidase (HRP) or lactoperoxidase (LPO) [3–8], no extensive comparative studies have yet been reported partly due to the difficulty in preparing a sufficient amount of purified TPO. Recent development of a rapid purification method [9] has made it possible to obtain a sufficient amount of the enzyme. Here, we compare the heme structures of the three peroxidases by means of vibrational spectroscopy.

Resonance Raman (RR) scattering from heme proteins reveals molecular vibrations of the heme group and allows detailed analysis of the heme structure [10]. In fact, RR studies have elucidated a difference between the heme structures of animal and plant peroxidases [11–14] and identified the sixth ligand of the heme iron in a reaction intermediate (compound II) of HRP with H_2O_2 [15–18]. Therefore, the RR spectra of TPO are expected to yield key information about its heme structure. Preliminary RR experiments indicated that the purified TPO preparation was strongly fluorescent in the oxidized state, and the fluorescence could not be easily depleted by further purification [7]. Therefore, although ordinary RR spectroscopy was used for the reduced form, for the oxidized species we adopted surface-enhanced RR spectroscopy (SERRS) [19], a technique which is based on the fact that adsorption of molecules to Ag colloid results in remarkable in-

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tensification of their Raman bands (~10^3-fold) and also in appreciable quenching of fluorescence. Since the possibility that adsorption of heme proteins to Ag colloid leads to structural changes in the heme group [20,21] cannot be ruled out, SERRS and ordinary RR spectra were recorded for LPO and HRP under the same experimental conditions as those for TPO for comparison.

2. MATERIALS AND METHODS

Hog TPO was purified by immunoaffinity chromatography [9]. The purified enzyme (M_r = 71000) had a specific activity of ~500 U/mg and gave an A_{418}/A_{280} ratio of 0.50–0.55. Bovine LPO was purified from cows milk as in [22]. Isozyme C of HRP was purchased from Toyobo (grade I-C, A_{403}/A_{280} = 3.2) and used without further purification. The enzymes were reduced using a small amount of solid sodium dithionite. The Ag colloid was prepared by reducing silver nitrate with sodium citrate according to Lee and Meise [23]. Ag particles in this preparation have a diameter of ~30 nm. This preparation was stable for several weeks at 0°C. The enzyme concentrations for measurements of SERRS and ordinary RR spectra were about 800 nM and 100 μM, respectively, in a solution of pH ~7.

The SERRS were excited by the 406.7 nm line of a Kr^+ laser (Spectra Physics, model 165) and detected by an OMA II system (PAR, 1215/1420) attached to a double monochromator (Spex 1401). The Ag colloid-adsorbed sample was spun at 0°C at 1800 rpm and the measurements were completed within 5 min after adsorption. The ordinary RR spectra of the enzyme solution were obtained using a Jeol 400D Raman spectrometer equipped with an RCA31034a photomultiplier. The reduced forms of the enzymes were excited by the 441.6 nm line of an He/Cd laser (Kinmon Electrics, CRD80MGH), while the oxidized forms were excited at 406.7 nm.

3. RESULTS AND DISCUSSION

Fig.1 shows the RR spectrum of native TPO and its SERRS. Native TPO emitted strong fluorescence (trace A), making analysis of its Raman spectrum impossible. When TPO was adsorbed to Ag colloid at a concentration of 800 nM, spectrum C was observed. Since Rayleigh scattering from colloidal particles gave an oblique background (trace B) under such slit-free conditions as adopted for the diode-array detection system, trace B was subtracted from spectrum C to obtain spectrum D. Hereafter, difference spectra such as spectrum D are referred to as SERRS.

Fig.2 shows the SERRS of resting HRP (A), LPO (B) and TPO (C). The spectral features of TPO resembled those of LPO but were clearly different from those of HRP. The highest frequency

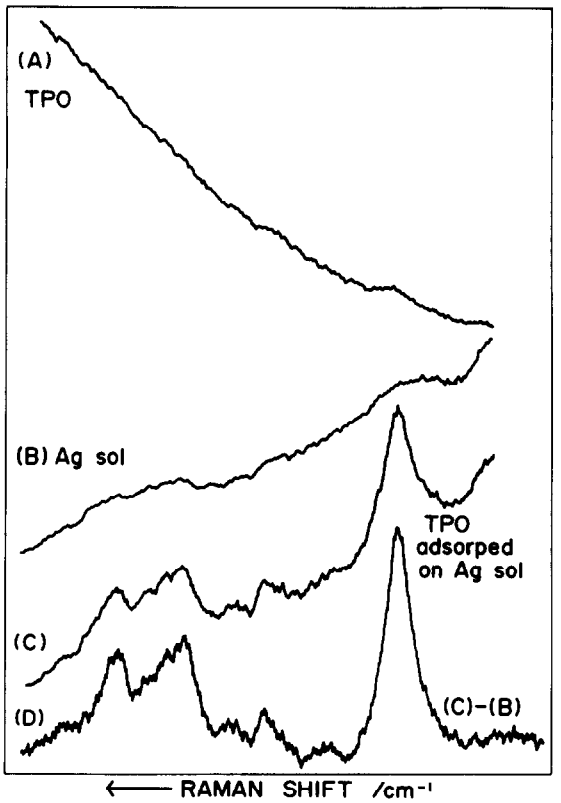

Fig.1. (A) Fluorescence background of resting TPO (40 M); (B) Raman spectrum of the Ag colloids; (C) SERRS of TPO adsorbed on the Ag colloids; (D) difference spectrum, (C) – (B). Excitation: 406.7 nm, 5 mW.

band in this region is assignable to the vinyl C=C stretching mode [24,25] and this frequency was higher for HRP than for LPO and TPO. The Raman band of the vinyl CH_2 scissoring mode, which is expected to appear around 1435 cm^{-1} [24,25], was observed for TPO and LPO but not for HRP. These facts suggest that the vinyl groups of TPO and LPO are more conjugated with the porphyrin macrocycle and thus more planar than those of HRP. The appearance of the vinyl bands strongly supports the presence of a protoheme in LPO and TPO, although the type of hemes contained in them has not necessarily been established as yet. We note that when the adsorbed TPO were desorbed from the Ag colloid by dilution at 5 min after adsorption, the enzymic activity was similar to that before adsorption.

In order to understand the implications of other RR bands, ordinary RR spectra were measured

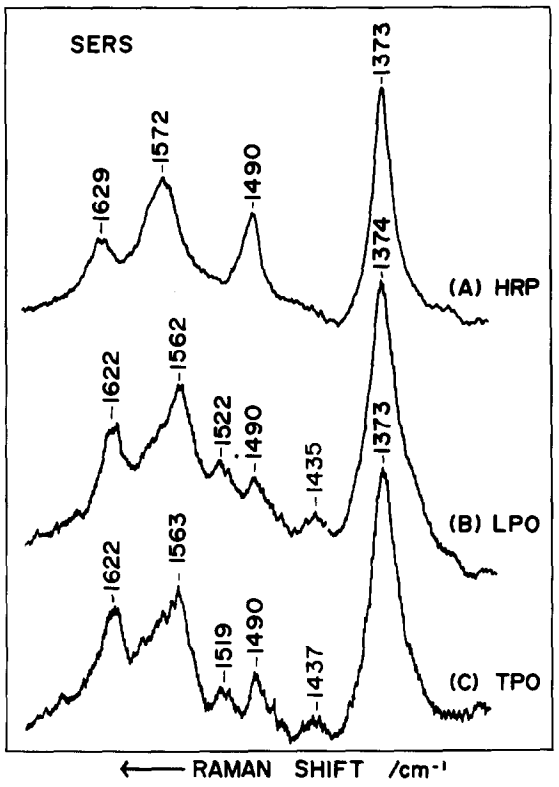

Fig.2. SERRS of resting HRP (A), resting LPO (B) and resting TPO (C) excited at 406.7 nm.

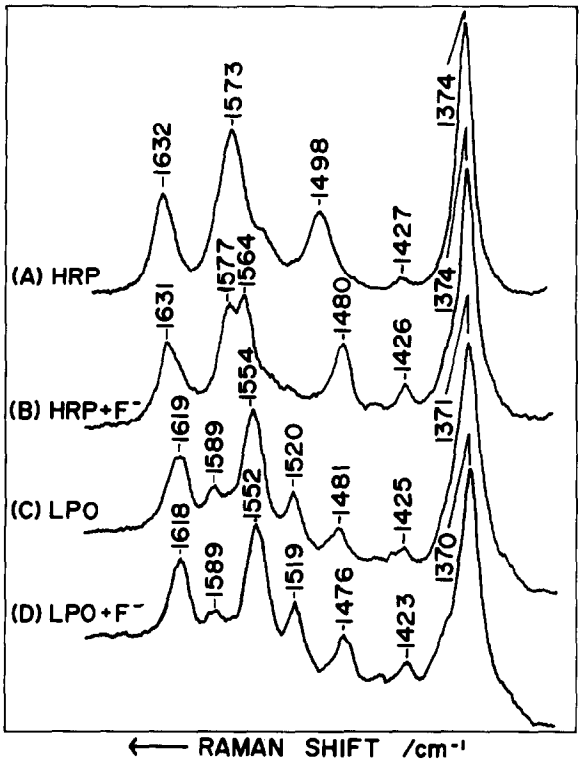

Fig.3. Ordinary resonance Raman spectra of HRP and LPO; (A) resting HRP, (B) fluoride adduct of HRP, (C) Resting LPO, (D) fluoride adduct of LPO.

with excitation at 406.7 nm for HRP and LPO as shown in fig.3. The Raman bands of HRP at 1573, 1498 and 1374 cm^{-1} (A) and those of LPO at 1554, 1481 and 1371 cm^{-1} (C) were polarized and accordingly assigned as ν_2, ν_3 and ν_4, respectively (mode number is based on [26]). On the basis of the empirical rule concerning the ν_3 frequency [27], the observed RR spectra imply that HRP and LPO adopt the five- and six-coordinate high-spin states, respectively, as suggested previously [11,13]. Indeed, when F^-, which is believed to bind to the sixth coordination position of the heme iron, was added, HRP exhibited large spectral changes (spectrum B) whereas those displayed by LPO were slight (spectrum D). The vinyl modes around 1630 and 1427 cm^{-1} remained unchanged upon change in coordination number. The Raman band of LPO at 1520 cm^{-1}, which was also observed in the SERRS shown in fig.2, was tentatively assigned to the infrared active ν_{39} mode (E_u) [28]. The appearance of such a forbidden mode implies reduction in symmetry from D_{4h} which is consistent with the strong conjugation of the vinyl groups with the porphyrin skeleton at asymmetrical positions.

We noticed that the ν_3 frequencies of adsorbed LPO and HRP (fig.2) were slightly different from those for the solutions (fig.3). This suggests that some structural change took place upon adsorption to the Ag colloid. Previously, a partial spin state change from low- to high-spin was noted for the adsorption of cytochrome *c* to the Ag colloid [21]. This change was reversible and resulted in a thermal spin equilibrium. A similar change might have occurred in the present case. However, judging from the overall similarity of the SERRS of TPO to that of LPO, we deduce that TPO adopts the six-coordinate state in the resting state at pH 7. This is consistent with the similarity in enzymic activities of the two enzymes [7].

Fig.4 shows the RR spectra of TPO, LPO and HRP in the reduced state. The spectral pattern of TPO was more similar to that of LPO than to that

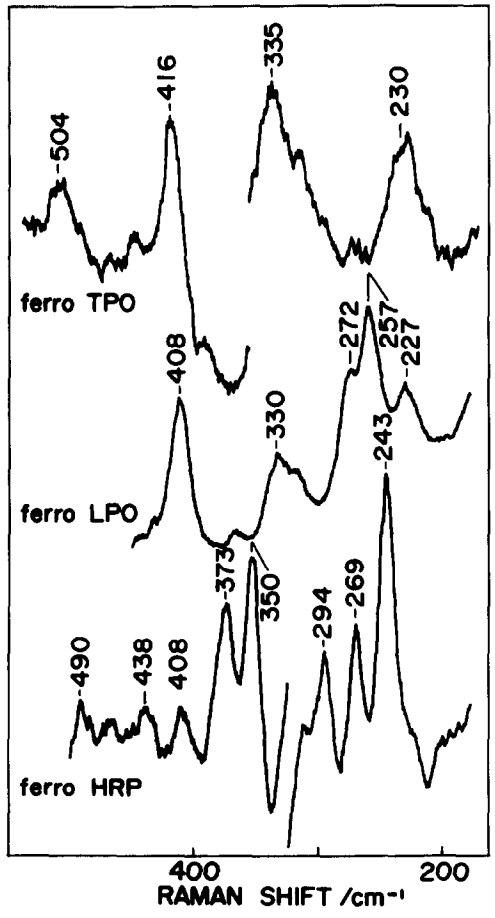

Fig.4. Resonance Raman spectra of reduced TPO (upper), reduced LPO (middle) and reduced HRP (bottom) excited at 441.6 nm (30 mW).

of HRP. However, the Fe^{2+}-histidine stretching mode ($\nu_{Fe\text{-}His}$), which was identified at 243 cm^{-1} for HRP [11] and 257 cm^{-1} for LPO [13], seems to be absent for TPO. Note that the $\nu_{Fe\text{-}His}$ RR band is observable for the five-coordinate high-spin state but not for the six-coordinate low-spin state [29]. Reduction of TPO gave a stable species with a Soret band at 433 nm, and this band remained unaltered for the Fe^{2+}-CN adduct with the six-coordinate low-spin state, suggesting that reduced TPO adopts the six-coordinate structure. The reduced form of LPO gave a derivative with a Soret band at either 435 or 446 nm and the 446 nm species was converted to the 435 nm form upon lowering of pH [8,14,30] or on formation of the CN^- adduct [8]. The $\nu_{Fe\text{-}His}$ band of reduced LPO at 257 cm^{-1} irreversibly diminished in intensity as with time elapsed during the Raman experiments with a concomitant reduction in intensity of the 446 nm absorption band. A similar spin transition from the five-coordinate high-spin to six-coordinate low-spin form was previously observed for hog intestinal peroxidase [12]. Consequently, it is very likely that reduced TPO adopts the six-coordinate structure and therefore the $\nu_{Fe\text{-}His}$ RR band was abolished.

In conclusion, both TPO and LPO contain an iron protoheme IX and the heme structure of TPO is close to that of LPO but differs from that of HRP. The difference is most prominent about the coordination number and the vinyl structure. The present study demonstrates that the SERRS technique is useful when the enzyme is fluorescent and present in small amounts.

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