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# Resonance Raman studies of the carbonmonoxy form of catalase

## Evidence for and effects of phenolate ligation

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Resonance Raman spectra are reported for the carbon monoxide (CO) adduct of catalase formed from the reaction of peracetic acid or hydrogen peroxide with the azide adduct of catalase in the presence of CO. The expected three normal vibrations of the Fe–CO fragment are detected at 1,908, 593 and 543 $cm^{-1}$ for the $\nu$(C–O), $\delta$(Fe–C–O) and $\nu$(Fe–CO), respectively. The expected coordination of the phenolate group in this adduct is confirmed by the enhancement of an internal vibration of phenolate, $\nu_{19a}$ at 1,515 $cm^{-1}$, and an extraordinary intensity enhancement of the $\nu$(Fe–CO) mode.

Resonance Raman; Carbon monoxide adduct; Catalase

## 1. INTRODUCTION

Catalases (EC 1.11.1.6, hydrogen peroxide:hydrogen peroxide oxidoreductase) are heme-containing enzymes which contain non-covalently bound iron protoporphyrin IX as their prosthetic group [1,2]. The enzyme is believed to protect the cellular components of aerobic organisms from oxidative respiratory byproducts by catalyzing the decomposition reaction of hydrogen peroxide to water and dioxygen. It also catalyzes the peroxidative oxidation of classical peroxidase substrates, albeit much less efficiently. An unusual structural aspect of catalase is its heme iron proximal ligand, which has now been firmly established, by high resolution X-ray crystal structural determinations [3–8], to be a phenolate group of tyrosine residues. Thus, it is of obvious interest to understand how the proximal phenolate ligation influences heme reactivity and thereby dictates catalase activity and distinguishes it from other heme enzymes possessing a proximal histidine (for example horseradish peroxidase) [9] or thiolate group (cytochromes *P*-450 and chloroperoxidase) [10,11].

Resonance Raman (RR) spectroscopy is well suited to probe the heme active-site structure [12] because of its intrinsic high sensitivity and selectivity. Excitation into the Soret or visible (Q band) heme $\pi$-$\pi^*$ transitions enhances not only the heme skeletal modes, but also the iron–axial ligand vibrations. Analysis of these spectral features has yielded a wealth of information regarding the structure and reaction mechanisms of various heme proteins. Previous RR works on catalases [13–16] have focused on the high frequency regions, particularly the vibrations arising from the proximal phenolate groups. Recently, this technique has been applied to study the reaction intermediates of catalase; i.e. Compounds I and II [17,18]. In this work, we report the RR characterization of an unusual form of catalase formed from the reaction of peracetic acid or hydrogen peroxide with azidocatalase in the presence of carbon monoxide (CO) [19]. We establish the essential nature of this derivative as the CO adduct of ferrous catalase by detecting the $\nu$(C–O) and $\nu$(Fe–CO) vibrations. The persistent coordination of the phenolate group in this adduct, confirmed by the enhancement of an internal vibration of phenolate, leads to extraordinary intensity enhancement of the $\nu$(Fe–CO) mode.

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## 2. EXPERIMENTAL

### 2.1. *Materials*

Catalase (C-100) was obtained from Sigma Chemical Co. (St. Louis, MO) and further purified by ion-exchange chromatography on DE-52 [17]. Fractions with a *Rz* value ($A_{400}/A_{280}$) greater than 0.88 were pooled and used in this work. The heme concentration of catalase was determined spectrophotometrically using an absorption coefficient $\varepsilon_{406} = 106\ mM^{-1}\cdot cm^{-1}$. Peracetic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI) and diluted to the desired concentration. The peracetic acid solution was pretreated with a trace amount of catalase overnight to decompose hydrogen peroxide [20]. The concentration of peroxides was determined spectrophotometrically just before use by using the method based on the lactoperoxidase-catalyzed oxidation of iodide [21].

2.2. *Preparation of the CO adduct of catalase*

This form of catalase was prepared according to previously described methods [19] with slight modification. Specifically, 0.5 ml of a solution of 50 μM catalase in a pH 7.0 buffer containing 20 mM potassium phosphate and 1 mM sodium azide was placed in a 5 mm o.d. NMR tube. The tube was then fitted with a septum and degassed with argon to remove oxygen. The solution was then exposed to either natural abundance or $^{13}$C-labeled CO. The desired product was formed by injection of a 10-fold excess of peracetic acid in the pH 7.0 buffer.

2.3. *Spectral measurement*

Resonance Raman spectra were acquired with a Spex 1403 spectrometer equipped with Hamamatsu R-928 photomultiplier and a Spex DM1B system controller. The 413.1 nm excitation line was obtained from a Coherent Innova Model 100-K3 $Kr^+$ ion laser. Throughout the spectral acquisition process, the sample was spun to avoid local heating by the laser beam.

## 3. RESULTS AND DISCUSSION

The addition of a 10-fold excess of peracetic acid to a solution of catalase and sodium azide in the presence of carbon monoxide results in the formation of a distinct species which exhibits electronic absorption bands at 427, 545 and 580 nm. The resonance Raman spectrum of this derivative is thus acquired with a 413.1 nm laser line in this work to achieve B-state resonance. Figure 1 displays the high frequency portion of the spectrum where skeletal vibrations of the heme macrocycle occur. These modes are very useful in monitoring the oxidation and spin state of the heme iron. The B-state excitation preferentially enhances the $A_{1g}$ modes, because of the dominance of the Franck-Condon mechanism [22]. In addition, the $B_{1g}$ and $B_{2g}$ modes can also be observed with weaker intensity, presumably owing to Jahn–Teller effects [22]. Thus, the bands observed between 1,300 and 1,700 cm$^{-1}$ can be assigned according to the nomenclature of Abe et al. [23,24] to the $A_{1g}$ mode: $\nu_2$ (1,588), $\nu_3$ (1,501) and $\nu_4$ (1,376); the $B_{1g}$ modes: $\nu_{10}$ (1,624) and $\nu_{11}$ (1,568); the $B_{2g}$ modes: $\nu_{28}$ (1,476) and $\nu_{29}$ (1,400); and the $E_u$ modes: $\nu_{37}$ (1,607) and $\nu_{38}$ (1,550 cm$^{-1}$). The RR activities of infrared-allowed $E_u$ vibrations are induced by the presence of the vinyl groups [25], which strongly perturb the electronic structure of heme macrocycle. The internal vibrations of the vinyl groups are also evident in the spectrum [26]. For example, the $\delta(=CH_2)$ is observed at 1,431 cm$^{-1}$. The 1,624 cm$^{-1}$ feature is probably a composite of both the $\nu$(C = C) and $\nu_{10}$, which, however, are separated in the spectrum of cytochrome catalase (trace B of Fig. 1).

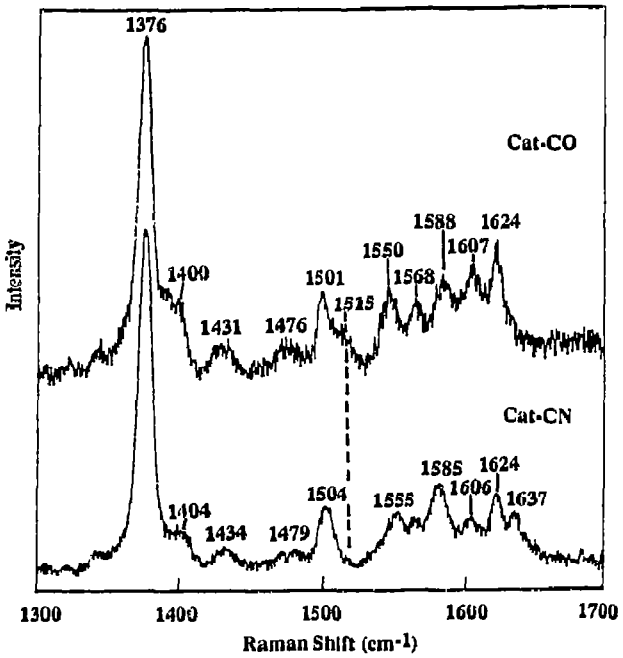


Fig. 1. High frequency resonance Raman spectrum of the CO and Cn adduct of catalase. Sample conditions: 100 μM catalase in 20 mM potassium phosphate buffer at pH 7.0. Excitation: 413.1 nm, 20 mw at sample, 0.5 cm$^{-1}$/0.5 s, 2 scans averaged.

Comparison of the RR spectra of the CO and $CN^-$ adducts of catalase reveals a unique feature at 1,515 cm$^{-1}$ for the CO complex. Considering that there is no candidate mode of heme skeletal vibrations in this region, we attribute the 1,515 cm$^{-1}$ band to the internal $\nu_{19a}$ vibration of the coordinated proximal tyrosylate. Several of the phenolate vibrations, particularly the ring $\nu$(C = C) modes at 1,612 and 1,510 cm$^{-1}$, have been previously observed in the RR spectra of heme proteins possessing proximal tyrosylate in the ferric state [14,15,27,28]. Although we are unable to unambiguously identify another expected phenolate mode at about 1,610 cm$^{-1}$, a band at 1,607 cm$^{-1}$ appears stronger than the corresponding one in the spectrum of the $CN^-$ adduct, suggesting a possible contribution from this mode. It is plausible that the phenolate-to-iron charge transfer transition is shifted beneath the strong Soret band upon the formation of the CO adduct.

To confirm the formation of a CO adduct of catalase under the reaction conditions, we recorded the RR spectrum in the region where $\nu$(C–O) and $\nu$(Fe–CO) vibrations are normally detected for other heme proteins. Fig. 2 documents the detection of the internal C–O stretching vibration of the bound CO. The 1,908 cm$^{-1}$ feature clearly downshifts to 1,863 cm$^{-1}$ upon $^{12}$CO/$^{13}$CO isotope substitution. The isotope shift (45 cm$^{-1}$) is similar to those reported for other systems [29] and agrees reasonably well with the calculated value (42.5 cm$^{-1}$), using a two-body vibrator model.

Fig. 3 shows the low frequency RR spectra of the natural abundance and $^{13}$C-labeled CO adduct of catalase. The $\nu$(Fe–CO) and $\delta$(Fe–C–O) modes are observed at 542 and 593 cm$^{-1}$, respectively. Their 3 and 12 cm$^{-1}$ downshifts upon the $^{13}$CO substitution support the assignment, which agrees with previous studies [29]. The unusually high $\nu$(Fe–CO) and low $\nu$(C–O) frequencies can be accounted for by the formation of strong distal side hydrogen bondings as is seen for many other peroxidases [30–32]. It should be pointed out that we ob-

served another band at 513 $cm^{-1}$ which showed a 6 $cm^{-1}$ downshift in the spectrum of the $^{13}CO$ adduct. This $\nu$(Fe–CO) mode may arise from a minor conformer of the bound CO, which is not hydrogen bonded to distal histidine. This spectroscopic behavior has been previously noted for the alkaline forms of the CO adducts of several peroxidases [30–32]. However, our attempts to prepare the CO adduct of catalase above pH 9 failed. Adjusting the pH of the CO adduct at pH 7.0 to above 9 resulted in the decomposition of the desired derivative.

We wish to call attention to the relative intensity of the $\nu$(Fe–CO) and $\nu_7$ mode at 678 $cm^{-1}$. As shown in Fig. 3, the intensity of the $\nu$(Fe–CO) band (542 $cm^{-1}$) is about 1.5 times that of the $\nu_7$ mode. A survey of the published RR spectra of the CO adducts of heme proteins and model compounds [33] indicates that the intensity of the $\nu$(Fe–CO) mode is at most about 0.1 of that of $\nu_7$. The only exceptions are those observed in the spectra of CO adducts of cytochromes *P*-450 [34], in which the proximal ligand is a thiolate group. It seems to suggest that the anionic character of phenolate in catalase (and thiolate in cytochromes *P*-450) may amplify the intensity of the $\nu$(Fe–CO) mode. However, in order to better understand the underlying enhancement mechanism, it is necessary to construct a detailed excitation profile. For example, the excitation profile of the $\nu$(Fe–S) mode in high spin state ferric cytochrome *P*-450cam has revealed a charge transfer absorption band on the high energy shoulder of the strong Soret band [35].

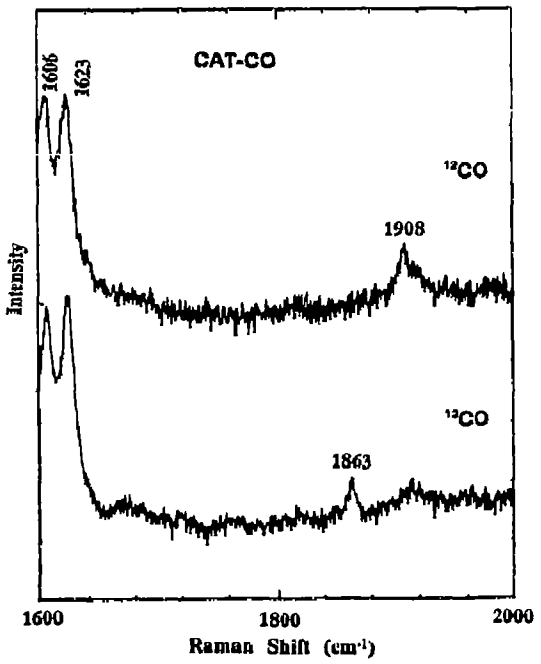


Fig. 2. Resonance Raman detection of $\nu$(C–O) mode of the CO adduct of ferrous catalase. Excitation: 413.1 nm, 0.5 $cm^{-1}$/0.5 s, 10 scans. Other conditions are the same as those in Fig. 1.

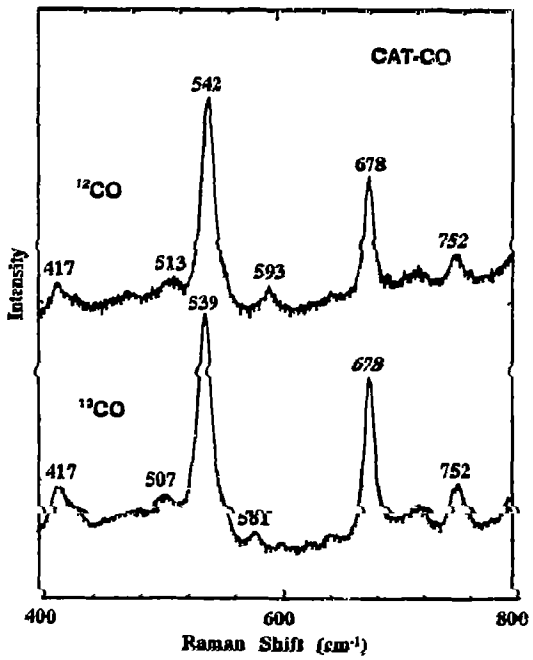


Fig. 3. Low frequency resonance Raman spectrum of the CO adduct of ferrous catalase. Excitation: 413.1 nm, 0.5 $cm^{-1}$/0.5 s, 3 scans. Sample conditions are the same as those in Fig. 1.

Tyrosylate ligation to the heme iron has been firmly established by X-ray crystal structure determination of catalase [3–8] and some abnormal hemoglobins [36,37] in their ferric state. The appearance of several phenolate vibrations in the corresponding RR spectra is consistent with this fact and predicts such a linkage in other catalases [15], abnormal hemoglobins [27,28] and genetically engineered sperm whale myoglobin [38] in which the proximal F8 histidine is replaced by a tyrosine. However, it has been suggested that the Fe–phenolate coordination is broken when these abnormal hemoglobins are reduced [38–40]. In fact, the Fe–CO vibrations in these CO adducts are found to be nearly the same as those of normal hemoglobin, implying the conversion to a histidine ligated form [38,39]. Given these facts and suggestions, the present work is the first report of a CO adduct of a heme protein or model compound in which the proximal ligand is phenolate.

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