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Identification of the Fe–O_2 and the Fe=O Heme Species for Indoleamine 2,3-Dioxygenase during Catalytic Turnover

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Resonance Raman spectroscopy has been applied to two distinct temporal species of indoleamine 2,3-dioxygenase during catalytic turnover. We have identified two oxygen-isotope-sensitive Raman modes at 569 and 798 cm^{-1} for the two respective species. The $^{16}O^{18}O$ analysis of the 798 cm^{-1} band indicates the existence of a ferryl-oxo heme, which is inconsistent with the previously proposed reaction mechanism. The present study thus provides a physical basis for the structures of the possible reaction intermediates.

Indoleamine 2,3-dioxygenase (IDO) is a heme-containing dioxygenase[1] localized in several different mammalian tissues, with the exception of liver.[2] This enzyme catalyzes a dioxygenation reaction for incorporation of two oxygen atoms from O_2 into L-tryptophan (Trp) in the production of *N*-formylkynurenine, which is the main catabolic pathway of Trp.[1] In the catalytic mechanism of IDO, a Fe–O–O–Trp type bridged intermediate has been postulated, subsequent to the primary oxygenated intermediate.[3] However, the catalytic mechanism remains controversial because the structures of the intermediates have not been characterized. In order to clarify the coordination geometry of the intermediate heme species, resonance Raman (RR) spectroscopy is potentially powerful. Recently, the oxygen-isotope-sensitive Raman bands have been reported for IDO in the presence and absence of Trp.[4,5] The RR bands at ca. 570 and ca. 800 cm^{-1} have been ascribed to be the Fe–O_2 and the Fe=O stretching modes based on the $^{18}O_2$ isotopic frequency shift.[5] However, in order to make definitive assignments of such modes, the Fe=O stretching mode in particular, the results of $^{16}O^{18}O$ experiments are crucial. In the present study, we examined the effect of $^{16}O^{18}O$ on the RR bands of the two distinct temporal species of IDO.

The absorption spectra of the two distinct species of IDO are shown in Figure S1.[6] Figure S1A[6] was obtained for the reduced IDO samples after addition of dioxygen. The spectrum exhibits absorption maxima at 413, 542, and 577 nm and is consistent with the reported spectra for oxygenated IDO.[7] The spectrum of Figure S1B[6] was obtained for oxygenated IDO samples after addition of Trp and exhibits absorption maxima at 410, 544, and 577 nm with decreased intensities relative to the absorption maxima of the oxygenated state. This spectrum also has a shoulder at 593 nm, and the intermediate represented in this spectrum, which has not hitherto been reported, is hereafter referred to as the "593 nm species." The absorption band at 593 nm is not seen in the absorption spectra of ferric and ferrous IDO (not shown), and the molar extinction coefficient at 593 nm is not known at present. If we assume that it is comparable to those of the α and β absorption bands of ferric and ferrous IDO, it is estimated that the fractional population of the 593 nm species seen in Figure S1B[6] is not smaller than 30%. We measured the time-dependent change of the concentrations of the 593 nm species and *N*-formylkynurenine during turnover. The results are shown in Figure S2.[6] When the time course of the absorption change at 321 nm (Figure S2B[6]) is compared with the time course of the absorption change at 593 nm (Figure S2C[6]), it becomes evident that *N*-formylkynurenine is generated when the 593 nm species is present, suggesting that it might be an intermediate.

Figure 1 depicts RR spectra for the oxygenated IDO as determined by simultaneously observed absorption spectra using the device shown in Figure S3.[6] Figure 1C shows that the Raman band at 569 cm^{-1} of the $^{16}O_2$ species exhibits a downshift to 541 cm^{-1} upon substitution with $^{18}O_2$. This represents a 28 cm^{-1} downshift. The inset of Figure 1 shows RR difference spectra using $^{16}O^{18}O$. If the band arises from the ν_{Fe-O2} mode of a side-on geometry, $^{16}O^{18}O$ should produce a band of an intermediate frequency at 555 cm^{-1}. However, Figure 1F shows that this is not the case. It is, therefore, demonstrated that the band at 569 cm^{-1} of the oxygenated IDO is arising from the ν_{Fe-O2} vibration of an end-on heme species. The position and the isotopic shift value of the Raman band at 569 cm^{-1} are close to the reported values of the ν_{Fe-O2} mode for oxygenated hemoproteins.[8] The frequency of the ν_4 mode of ferric IDO was found to be 1373 cm^{-1} (not shown). The frequencies of the ν_4 mode at 1375 cm^{-1} and the ν_{10} mode at 1639 cm^{-1} in Figure 1 are consistent with an O_2-bound low-spin heme.

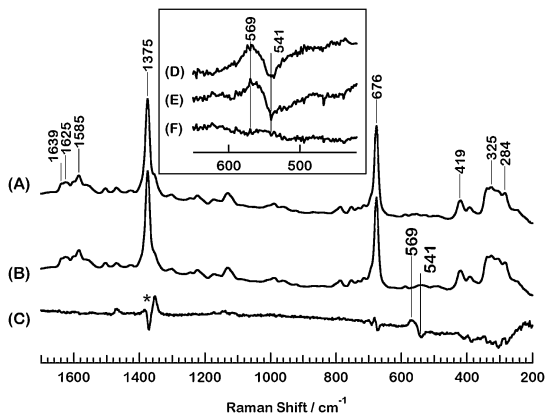

Figure 1. RR spectra of oxygenated IDO. (A) $^{16}O_2$, (B) $^{18}O_2$, (C) ($^{16}O_2$–$^{18}O_2$) × 5, (D) $^{16}O_2$–$^{16}O^{18}O$, (E) $^{16}O^{18}O$–$^{18}O_2$, and (F) $^{16}O^{18}O$–($^{16}O_2$ + $^{18}O_2$)/2.

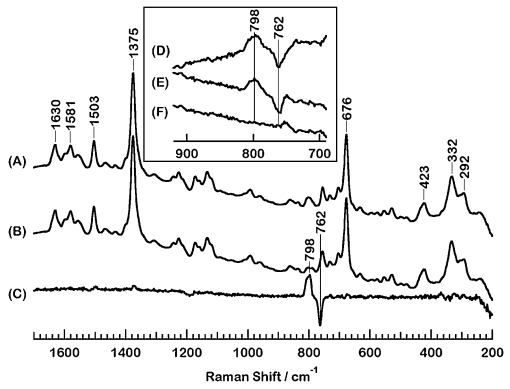

Figure 2. RR spectra of the 593 nm form of IDO. (A) $^{16}O_2$, (B) $^{18}O_2$, (C) ($^{16}O_2$–$^{18}O_2$) × 5, (D) $^{16}O_2$–$^{16}O^{18}O$, (E) $^{16}O^{18}O$–$^{18}O_2$, and (F) $^{16}O^{18}O$–($^{16}O_2$ + $^{18}O_2$)/2.

Figure 2 depicts RR spectra of the 593 nm species as determined by simultaneously observed absorption spectra. The Raman bands at 569 and 541 cm^{-1} of the oxygenated species in Figure 1C are completely replaced by bands located at 798 and 762 cm^{-1} in Figure 2C. The 798 cm^{-1} band could be assigned as either the $\nu_{Fe=O}$ vibration of an oxo-iron species[9] or the ν_{OO} vibration of a peroxide adduct (Fe–O_2^{2-}, either an end-on[10] or a side-on species[11]). If the band is due to the ν_{OO} mode, the $^{16}O^{18}O$ adduct should exhibit an intermediate frequency of approximately 780 cm^{-1}. The inset of Figure 2 shows the results using $^{16}O^{18}O$. Figure 2F demonstrates that $^{16}O^{18}O$ does not produce the intermediate frequency band. The results thus indicate that the Raman band at 798 cm^{-1} for the 593 nm species is due to the $\nu_{Fe=O}$ vibration of a Fe=O heme species. We tentatively assign the formal charge of the heme iron of the 593 nm species of IDO to be Fe^{4+} (a ferryl-oxo species) based on many reported examples.[9] The frequency of the ν_4 mode at 1375 cm^{-1} in Figures 2A and 2B compared with 1373 cm^{-1} for ferric IDO (not shown) is consistent with the existence of Fe^{4+} heme.

Figure S4[6] shows RR spectra for mixed-flow single turnover experiments. Figures S4C and S4F[6] exhibit Raman bands at ca. 570/540 cm^{-1} and ca. 800/760 cm^{-1}, respectively, for $^{16}O_2$/$^{18}O_2$ isotopes. The positions of these bands are essentially identical to those of the Fe–O_2 species (Figure 1C) and the Fe=O species (Figure 2C). These results suggest that the oxygenated intermediate is converted to the ferryl-oxo species upon reaction with Trp.

As mentioned above, in the previously proposed molecular mechanism, it has been considered that the two oxygen atoms are incorporated into Trp without cleavage of the O–O bond.[3] If the ferryl-oxo species detected in the present study is definitely involved in the reaction, an important consequence is that the O–O bond of dioxygen is cleaved before the oxygen atoms are incorporated into Trp. Certain theoretical calculations are consistent with the present results,[5,12] but others are not in agreement.[13] While further study is needed to clarify the reaction mechanisms, this study has conclusively demonstrated the coordination geometry of the two distinct temporal species of IDO and has, therefore, provided a physical basis for the structures of the possible reaction intermediates.

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