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Rapid Report

Identification of the iron-carbonyl stretch in distal histidine mutants of carbonmonoxymyoglobin

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Abstract

Soret-excitation resonance Raman (RR) spectra are reported for six distal histidine mutants of carbonmonoxymyoglobin including H64A, H64V, H64L, H64I, H64W, and H64W/L29F. Based on ^{13}CO isotope shifts, the iron-carbonyl stretching vibrations are unambiguously identified. The correct assignment of these modes eliminates the differences in the conformational substate occupations predicted by the RR versus IR data.

Keywords: Myoglobin; Genetic modification; Resonance Raman; Infrared; Conformational substate

The Fe-CO unit of carbonmonoxymyoglobin (MbCO) has been extensively examined by infrared (IR) and resonance Raman (RR) spectroscopy [1–8]. IR studies have focused on the carbonyl stretching vibration, $\nu_{C\text{-}O}$, of the bound ligand and have shown that in mammalian Mbs, there are multiple Fe-CO conformers (designated as A states, with subscripts numbered in descending order of the carbonyl stretching frequency, $\nu_{C\text{-}O} = 1965$ (A_0), ~ 1945 ($A_{1,2}$), and 1932 (A_3) cm^{-1}) [1–5]. In the RR spectrum, the iron-carbonyl stretching, $\nu_{Fe\text{-}CO}$, and bending, $\delta_{Fe\text{-}CO}$, vibrations are observed in addition to $\nu_{C\text{-}O}$ [6–8]. RR and IR studies on MbCO, as well as on the CO complexes of a large number of iron porphyrins and other heme proteins, have shown that $\nu_{Fe\text{-}CO}$ is inversely correlated with $\nu_{C\text{-}O}$ [9–12]. For MbCO, $\nu_{Fe\text{-}CO} = 491$ (A_0), ~ 508 ($A_{1,2}$), and 518 (A_3) cm^{-1} [13–16].

Under physiological conditions, native mammalian MbCOs are a mixture of conformers $A_{1,2}$ (70%) and A_3 (30%) [1–5]. The relative populations of the A states can be altered by changing the pH, temperature, or ionic strength [13–20]. Recent studies of genetically modified MbCOs have shown that the A-state populations are also sensitive to the nature of the amino acid residues in the heme pocket [13–24]. IR studies of sperm whale, human, and porcine MbCOs in which the distal histidine, H64 (E7), has been replaced with aliphatic amino acids such as valine, leucine, and isoleucine have shown that $\nu_{C\text{-}O}$ is a relatively narrow Lorentzian contour centered near 1966 cm^{-1} [19,24]. In the case of the alanine mutant, the principal $\nu_{C\text{-}O}$ band is also near 1966 cm^{-1}; however, weak shoulders are observed at lower energies. These observations suggest that H64V, H64L, and H64I are exclusively in the A_0 state and that H64A is predominantly in this state [19,24].

In contrast to the single conformational-state structure suggested by the IR data, the RR spectra of aliphatic mutants of porcine and human MbCOs suggest the presence of multiple Fe-CO conformers. The RR spectrum of porcine H64V is characterized by five bands in the $\nu_{Fe\text{-}CO}$ region (475–525 cm^{-1}) [21,22]. The principal band is near 491 cm^{-1}, consistent with the population of A_0 indicated by the IR data. However, a secondary RR band with significant intensity is observed near 508 cm^{-1} along with much weaker components near 479, 496, and 518 cm^{-1}. Five RR bands with similar frequencies have also been reported in the RR spectra of several other porcine mutants [22]. All five RR bands in the aliphatic (and other) porcine mutants have been attributed to $\nu_{Fe\text{-}CO}$ modes arising from different Fe-CO conformers. In the case of the

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aliphatic mutants of human MbCO, the 490 and 508 cm^{-1} bands have been attributed to $\nu_{Fe\text{-}CO}$ modes arising from population of the open (A_0) and closed (A_1) forms of the protein, respectively [23]. The temporal behavior of these bands was used to monitor the relative rates of geminate recombination of CO to the two forms subsequent to laser photodissociation of the ligand.

The differences in the A-state populations predicted on the basis of the IR versus RR data of the aliphatic MbCO mutants is puzzling. The fact that differences are observed suggests that the spectral signatures of one or both techniques do not accurately reflect these populations. If so, the conclusions drawn from a considerable body of studies must be reevaluted. It must be noted, however, that the correct prediction of the A-state populations is contingent on the accurate assignment of the spectral features. The assignment of bands due to ν_{CO} is relatively straightforward because these bands fall in an uncongested region of the vibrational spectrum [6]. In contrast, the bands due to $\nu_{Fe\text{-}CO}$ fall in a region in which heme skeletal vibrations also contribute [6,7,25]. In order to distinguish $\nu_{Fe\text{-}CO}$ from these latter vibrations, studies must be conducted with isotopically labeled CO. Surprisingly, no spectral studies of MbCO mutants have been reported which utilize CO isotopomers [1].

In order to gain a better understanding of the relationship between the features observed in the IR and RR spectra of mutant Mbs, we performed a careful examination of the RR spectra of the aliphatic mutants H64A, H64V, H64L, and H64I of sperm whale MbCO. These studies utilized both normal and isotopically labeled CO. We also examined the RR spectra of two aromatic mutants, H64W and H64W/L29F. RR spectra have not been previously reported for these mutants. IR studies on H64W and H64W/L29F suggest that H64W is a mixture of A_0 and A_3 conformers (60/40%), whereas H64W/L29F, like the single L29F mutant, is almost exclusively in the A_3 form ($\sim$ 90%) [24] [2]. The RR data for these six mutants provide new insights into the spectral characteristics of the Fe-CO unit and resolve the apparent inconsistencies between the IR and RR data for the aliphatic mutants.

The mutant Mbs were prepared, isolated, and purified as described in Ref. [24] (and references therein). The MbCOs were prepared flushing the deoxy forms with CO under rigorously anaerobic conditions. The ambient temperature RR measurements were made on samples contained in a quartz spinning cell to prevent photoreduction (this was judged negligible as measured by the absence of any discernible contribution from the ν_4 mode of the photoproduct). The spectra were acquired by using a triple spectrograph (Spex 1877) equipped a liquid nitrogen cooled 1152×298 pixel charge-coupled device (Princeton Instruments LN/CCD with an EEV chip) as the detector. The excitation wavelengths were provided by the outputs of a Kr ion laser (Coherent Innova 200-K3) or continuous-wave dye laser (Coherent 599) utilizing Stilbene 420 (Exciton) pumped with the UV output of an Ar ion laser (Coherent Innova 400–15UV).

The Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of the ^{12}CO and ^{13}CO adducts of H64A, H64V, H64L, and H64I are shown in Fig. 1. RR spectra were also acquired at several other excitation wavelengths spanning the Soret band contour. The features observed at all excitation wavelengths in the 405–430 nm region are similar to those shown although the relative intensities of the RR bands change somewhat with λ_{ex}. The RR spectra of all four mutants are similar to one another and similar to those previously reported for the porcine and human aliphatic mutants [21–23]. In particular, at least four bands are observed: (1) a strong feature near 490 cm^{-1}, (2) a prominent secondary band near 505 cm^{-1}, and (3) two weak shoulders near 479 and 517 cm^{-1}. The fifth RR band reported for porcine H64V near 496 cm^{-1} is not readily apparent; however, simulations indicate that a weak feature may occur in this region. Upon substitution of ^{13}CO, the 490 cm^{-1} band of all four aliphatic mutants downshifts by 3–4 cm^{-1}. Concomitantly, the 573 cm^{-1} band downshifts by 16–17 cm^{-1}. These isotope shifts are similar to those previously reported for $\nu_{Fe\text{-}CO}$ and $\delta_{Fe\text{-}CO}$ of native sperm whale MbCO and identify these modes in the mutants [8,13]. High-frequency RR spectra of H64L (not shown) indicate that the 1966 cm^{-1} $\nu_{C\text{-}O}$ band is a narrow feature, and downshifts by 46 cm^{-1} upon isotopic substitution. None of the other RR bands in the 475–525 cm^{-1} region appear to exhibit any isotope shift. Isotope difference experiments and spectral simulations (not shown) confirm that the spectra are best accounted for in terms of a single isotope sensitive band [3]. These observations indicate that the other RR bands observed in the 475–525

[1] Isotope studies are mentioned in Ref. [23]; however, no spectral data were presented.

[2] In Ref. [24], H64W is described as a mixture of A_0 and $A_{1,2}$ states with $\nu_{C\text{-}O}$ near 1965 and 1942 cm^{-1}, respectively. However, $\nu_{C\text{-}O}$ for the latter conformer is at 517 cm^{-1}, which is more consitent with an A_3-like conformer.

[3] In the case of H64A, an additional weak isotope-sensitive feature underlies the non-shifting 504 cm^{-1} mode. This is evidenced by the fact that the trough btween the 490 and 504 cm^{-1} bands of H64A fills in slightly in the ^{13}CO adduct and the remaining feature at 504 cm^{-1} loses intensity. In the other three aliphatic mutants, the trough deepens and the intensity of the unshifting 504 cm^{-1} band remains constant.

cm^{-1} region are due to vibrations of the porphyrin ring rather than to $\nu_{Fe\text{-}CO}$ modes of different Fe-CO conformers. Collectively, the RR data for the aliphatic mutants are consistent with exclusive population of A_0 (except for H64A which exhibits minor amounts of $A_{1,2}$) as was previously proposed from the IR studies [19,24].

The Soret-excitation RR spectra of the ^{12}CO and ^{13}CO adducts of H64W/L29F and H64W are shown in

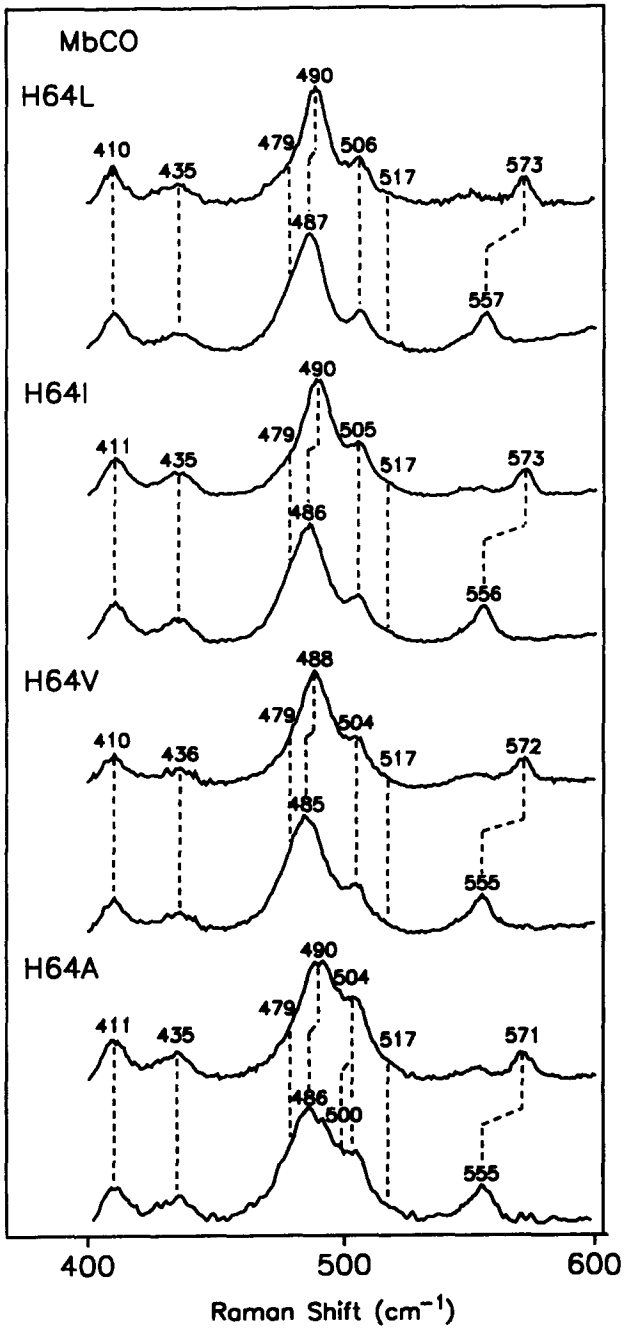

Fig. 1. Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of the aliphatic mutants of sperm whale MbCO. The ^{12}CO and ^{13}CO adducts for each mutant are shown in the upper and lower traces, respectively. All proteins were 50–100 mM in 0.1 M, pH 7.0 phosphate buffer. The laser power was typically 3 mW.

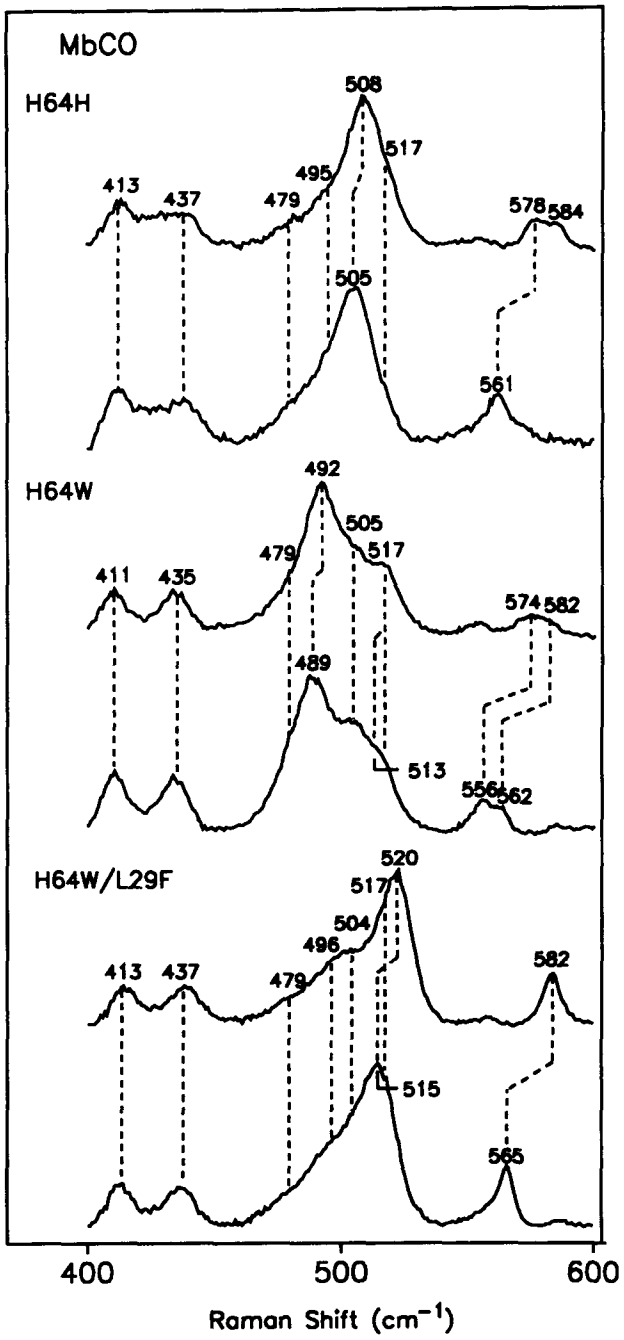

Fig. 2. Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of the aromatic mutants of sperm whale MbCO (bottom and middle panels). The analogous spectra of wild-type are shown in the upper panel. The ^{12}CO and ^{13}CO adducts for each protein are shown in the upper and lower traces, respectively. All other conditions are the same as in Fig. 1.

Fig. 2. As is the case for the aliphatic mutants, RR spectra acquired at other excitation wavelengths in the Soret absorption region are similar to those shown. The RR spectrum of H64W/L29F is dominated by a strong band near 520 cm^{-1} with additional features observed near 504, 496, and 479 cm^{-1}. The spectrum of the latter exhibits three prominent features near

517, 505, and 492 cm^{-1} plus a weak band near 479 cm^{-1}. In the ^{13}CO adducts, the single 520 cm^{-1} band of H64W/L29F downshifts by ~5 cm^{-1}, whereas both the 517 and 492 cm^{-1} bands of H64W downshift by 3–4 cm^{-1}. The isotopic shifts of the 520 and 517 cm^{-1} bands in the two mutants also uncover a weak unshifting feature near 517 cm^{-1}. For both mutants, none of the other spectral features in the 475–525 cm^{-1} region appear to shift upon isotopic substitution. Isotope difference experiments and spectral simulations (not shown) again confirm that the spectra are best accounted for if this is the case. These isotope data identify the 520 cm^{-1} band of H64W/L29F and the 517 and 492 cm^{-1} bands of H64W as $\nu_{Fe\text{-}CO}$ vibrations. Again, the 479, 496, 505, and unshifted 517 cm^{-1} features observed in the spectra of both mutants are due to porphyrin modes. Isotopic substitution also downshifts the bands in the 575–585 cm^{-1} region by 17–20 cm^{-1} identifying these as $\delta_{Fe\text{-}CO}$. In H64W/L29F, $\delta_{Fe\text{-}CO}$ is a single band (as is the case for all the aliphatic mutants), whereas two isotope-sensitive features are observed in this spectral region for H64W. This latter observation is consistent with the presence of two $\nu_{Fe\text{-}CO}$ bands. Collectively, the RR spectra indicate that H64W/L29F is mostly in the A_3 state, whereas H64W is a mixture of A_0 and A_3 conformers. This picture is again consistent with that proposed from IR studies [24].

The RR data reported herein indicate that four porphyrin bands occur in the vicinity of $\nu_{Fe\text{-}CO}$, most notably the 505 cm^{-1} band. The porphyrin bands observed for the mutant MbCOs are undoubtedly the analogs of bands that have previously been identified in the RR spectra of other ligand-bound Mbs (such as the O_2 and NO adducts) [8,25]. These observations suggest that the porphyrin modes should also contribute to the RR spectra of native (and wild-type) MbCO. For comparison, the Soret-excitation RR spectra of the ^{12}CO and ^{13}CO adducts of wild-type MbCO are included in the upper traces of Fig. 2. Because wild-type MbCO is predominantly in the $A_{1,2}$ state [1–16], the 508 cm^{-1} $\nu_{Fe\text{-}CO}$ band obscures the 505 cm^{-1} porphyrin band in both the ^{12}CO and ^{13}CO adducts. [4] However, the other porphyrin bands are clearly apparent towards lower wavenumbers. It should also be noted that the RR spectra of the $C^{18}O$ and $^{13}C^{18}O$ isotopomers of native sperm whale MbCO have been previously reported [8]. The shifts of $\nu_{Fe\text{-}CO}$ are larger for these isotopomers and inspection of these data (Ref. [8], Fig. 6) clearly suggests the presence of an additional band with substantial intensity underlying the isotope sensitive $\nu_{Fe\text{-}CO}$ band. The origin of this underlying mode was not discussed; however, a likely candidate is the 505 cm^{-1} porphyrin band observed in this study. The spectral data reported for these latter MbCOs along with that for the $C^{18}O$ and $^{13}C^{18}O$ isotopomers of native sperm whale MbCO suggest that this porphyrin mode may contribute as much as 30% of the intensity to the 508 cm^{-1} composite band contour normally attributed exclusively to $\nu_{Fe\text{-}CO}$. Accordingly, this porphyrinic contribution must be considered in any quantitation of the intensity of $\nu_{Fe\text{-}CO}$.

[4] The $\nu_{Fe\text{-}CO}$ mode of the A_3 form of wild-type, expected near 520 cm^{-1}, is not clearly observed. This band is presumably weak because the Raman cross-section for the A_3 form is very low [14]. Nevertheless, the RR intensities of the $\nu_{Fe\text{-}CO}$ modes of the A_3 forms of both H64W and H64W/L29F are substantial. This observation suggests that the low cross-section observed for the $\nu_{Fe\text{-}CO}$ mode of the A_3 form of native (and wild-type) is not a universal characteristic of A_3 forms.

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