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The Millisecond Intermediate in the Reaction of Nitric Oxide with Oxymyoglobin is an iron(III)-Nitrato Complex, not a Peroxynitrite

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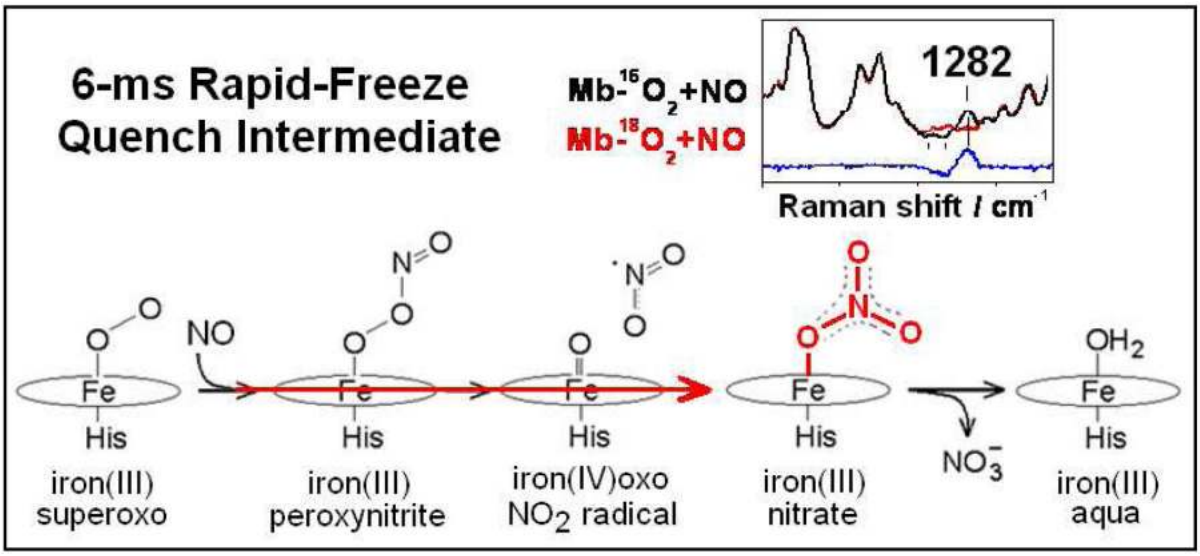
Abstract



The dioxygenation of nitric oxide by oxyheme in globin proteins is a major route for NO detoxification in aerobic biological systems. In myoglobin, this reaction is thought to proceed through an iron(III)-bound peroxynitrite before homolytic cleavage of the O-O bond to form an iron(IV)-oxo and NO_2 radical followed by recombination and nitrate production. Single turnover experiments at alkaline pH have revealed the presence of a millisecond high-spin heme intermediate. It is widely presumed that this species is an iron(III)-peroxynitrite species, but detailed characterization of the intermediate is lacking. Using resonance Raman spectroscopy and rapid-freeze quench techniques, we identify the millisecond intermediate as an iron(III)-nitrato complex with a symmetric NO_2 stretch at 1282 cm^{-1}. Greater time resolution techniques will be required to detect the putative iron(III) peroxynitrite complex.

Nitric oxide (NO) is an important signaling molecule in mammals, influencing such diverse functions as immune response, vasodilation, and smooth muscle contraction among others.[1] While submicromolar NO concentrations are sufficient to perform these signaling functions, higher concentrations are toxic, via inhibition of essential enzymes including metalloenzymes

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from the respiratory chain.[2] In aerobic environments, NO's cytotoxicity is associated with its near diffusion-limited reaction rate with superoxide to generate peroxynitrite.[3] Myoglobin and hemoglobin have long been recognized for their role in O_2 storage and delivery, respectively. More recently, oxymyoglobin (Mb-O_2) and oxyhemoglobin have been shown to be potent sinks for NO *in vivo*.[4, 5] Mb-O_2 converts NO to nitrate (NO_3^-) with complete retention of the isotopic constitution of the O_2 and NO reactants. The mechanism is presumed to proceed via a high-spin ferric peroxynitrite intermediate before homolytic cleavage of the O-O bond and recombination of the NO_2 radical with the ferryl-oxo to form the nitrate product (Figure 1). The same reaction mechanism is likely to be the source of the NO dioxygenase activity reported in bacterial flavohemoglobins.[5, 6]

There are no observable intermediates in this reaction at neutral pH, but stopped-flow UV-vis experiments at alkaline pH show the rapid buildup and decay of a high-spin ferric species.[7, 8] Rapid-freeze quench (RFQ) samples of the reaction analyzed by EPR spectroscopy also support the formation of a ferric high-spin transient species.[9] For myoglobin at pH 9.5 and 20° C, stopped-flow results indicate that the build-up in high-spin intermediate is maximal within the dead-time of the instrument (1 to 3 ms) and decays fully within 15 to 20 ms. On the basis of the UV-vis characteristics of the intermediate, the species was assigned to an iron(III)-peroxynitrite complex.[7, 8] Here, we report the first characterization of this millisecond intermediate by resonance Raman spectroscopy and identify this species as an iron(III)-nitrato complex rather than an iron(III)-peroxynitrite complex.

Resonance Raman (RR) spectra obtained with Soret excitation are highly sensitive to the oxidation, spin, and coordination states of the heme iron.[10] This technique is an ideal analytical tool for studying the NO dioxygenation reaction since both starting Mb-O_2 and final met-Mb are six-coordinate low-spin species at low temperature and alkaline pH, while the putative intermediate is a six-coordinate high-spin species. Figure 2 shows the high-frequency RR spectra of RFQ samples taken at different time points during the reaction of Mb-O_2 with NO at pH 9.5 and 3° C. The RFQ sample with the shortest reaction time is trapped within 6 ms (see Supporting Information for experimental details). Its RR high-frequency spectrum exhibits strong ν_2 and ν_3 porphyrin skeletal modes at 1566 and 1482 cm^{-1}, characteristic of six-coordinate high-spin vibrations, while their low-spin counterparts[11] at 1585 and 1505 cm^{-1} are weak. In contrast, the RR spectra of RFQ samples with longer reaction times show weak high-spin features, and the gradual conversion of high-spin to low-spin species is essentially complete by 55 ms (Figure 2).

RFQ samples of the reaction of Mb-$^{16}O_2$ and Mb-$^{18}O_2$ with ^{14}NO, ^{15}NO, or $^{15}N^{18}O$ were used to isolate vibrational modes involving the exogenous ligand. RR spectra of the 6-ms RFQ samples of these reactions reveal a mode at 1282 cm^{-1} that downshifts with Mb-$^{18}O_2$ and is observed at 1260 cm^{-1} in Mb-$^{16}O_2$ + ^{15}NO (Figure 3). This RR band is not observed in the RR spectra of samples trapped after longer reaction times, and accordingly, it is assigned to a ligand vibration from the high-spin ferric millisecond intermediate species (Figure 3). As expected, the low-frequency RR spectra of Mb-O_2 exhibit a ν(Fe-O_2) at 577 cm^{-1} that shifts −26 cm^{-1} with $^{18}O_2$, but no isotope sensitive modes could be detected in the low-frequency RR spectra of the 6-ms RFQ samples (Figure S1). Additional testing of these samples with 351- and 458-nm laser excitations on either side of the Soret absorption did not enhance any new isotope sensitive vibrations (data not shown).

The frequency and isotope sensitivity of the 1282-cm^{-1} signal are not readily matched with expectations for peroxynitrite vibrations. [12] Instead, the 1282-cm^{-1} Raman band is consistent with a $\nu_s(NO_2)$ from an iron(III)-nitrato complex. For example, the six-coordinate high-spin Fe(TPP)(η^1 -ONO_2)(THF) complex shows FTIR spectra with a strong $\nu_s(NO_2)$ at 1280 cm^{-1} that shifts −22 cm^{-1} with ^{15}N.[13] The assignment of the 1282-cm^{-1} signal to a nitrato

$\nu_s(NO_2)$ in the RR spectra of the 6-ms intermediate is further supported by the impact of ^{18}O-labeling of Mb-O_2 and NO. Indeed, labeling of all three O-atoms produces the largest downshift of this mode to 1232 cm^{-1} (Figure S2 shows the RR spectrum of Mb-$^{18}O_2$ + $^{15}N^{18}O$). In contrast, when Mb-$^{18}O_2$ is reacted with $N^{16}O$, two $\nu_s(NO_2)$ frequencies are observed (Figure 3): the band with the largest downshift corresponds to Fe-$^{16}ON^{18}O_2$ and the other to Fe-^{18}ON (^{16}O)^{18}O. While the FTIR spectra of Fe(TPP)(η^1-ONO_2)(THF) show a weak $\nu_{as}(NO_2)$ at 1491 cm^{-1} and a very weak ν(N-O) near 1000 cm^{-1}, [13] these same modes are also expected to be weak in RR, and it is not surprising that they are not observed in the RR spectra of the 6-ms RFQ samples.

Although our results do not support the proposed peroxynitrite assignment by Herold and coworkers,[8] the trapping of an iron(III)-nitrato complex is not inconsistent with a recent theoretical study by Blomberg and coworkers.[14] Using density functional theory (DFT), the decay of the peroxynitrite transient via homolytic cleavage of the O-O bond was predicted to occur with an energy barrier insufficient for accumulation of this species in the course of the reaction. Moreover, attempts to model the stabilization of a high-spin millisecond intermediate at alkaline pH as a peroxynitrite, via distal or proximal perturbations, were unsuccessful.[14] In contrast, because anionic ligands show decreasing dissociation rate constants with increasing pH,[15] stabilization of the iron(III)-nitrato complex is expected at alkaline pH, and is supported by our results. The assignment of the ms-intermediate to an iron(III)-nitrato complex was rejected by Herold and coworkers on the basis of differences between the absorption spectrum of the ms-intermediate and resting Mb with nitrate.[8] However, a very large excess of nitrate (ca. 10^5 equiv) is required to affect the UV-vis spectrum of metMb and it is unclear whether the spectral changes reflect coordination of nitrate to the heme iron(III), or less specific conformational changes. Control RFQ-RR experiments with metMb and high concentrations of nitrite or nitrate at pH 9.5 did not reveal formation of any high-spin heme species or vibrations involving exogenous ligands.

In conclusion, our RR analysis of RFQ samples of the reaction of oxy-Mb + NO at pH 9.5 identifies the ms-intermediate as an iron(III)-nitrato complex. It is important to stress that this species is not observed under equilibrium conditions, even with a high excess of nitrate, and thus corresponds to a transient iron(III) complex. Assigning the ms-intermediate species to a nitrate rather than a peroxynitrite complex resolves the discrepancy between theoretical and experimental studies of the NO dioxygenation reaction in Mb. Our results do not invalidate the proposed reaction mechanism shown in Figure 1. Rather, they indicate that a greater time resolution will be required to determine whether a heme-peroxynitrite intermediate forms in the course of this reaction, and to structurally characterize this species. Such experiments with a microsecond freeze-quench apparatus[16] are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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References

1. (a) Moncada S, Palmer RM, Higgs EA. Pharmacol. Rev 1991;43:109–142. [PubMed: 1852778] Lancaster, J, Jr. Nitric oxide: Principles and actions. San Diego: Academic press, inc.; 1996. (c) Fukuto JM, Wink DA. Met. Ions Biol. Syst 1999;36:547–595. [PubMed: 10093936]

2. (a) Fang FC. J. Clin. Invest 1997;99:2818–2825. [PubMed: 9185502] (b) Nathan C, Shiloh MU. Proc. Natl. Acad. Sci. U. S. A 2000;97:8841–88418. [PubMed: 10922044] (c) Gardner PR, Martin LA, Hall D, Gardner AM. Free Radic. Biol. Med 2001;31:191–204. [PubMed: 11440831]
3. (a) Huie RE, Padmaja S. Free Radic. Res. Commun 1993;18:195–199. [PubMed: 8396550] (b) Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Proc. Natl. Acad. Sci. U. S. A 1996;93:6770–6774. [PubMed: 8692893] (c) Kissner R, Nauser T, Bugnon P, Lye PG, Koppenol WH. Chem. Res. Toxicol 1997;10:1285–1292. [PubMed: 9403183]
4. (a) Poole RK, Hughes MN. Mol. Microbiol 2000;36:775–783. [PubMed: 10844666] (b) Brunori M. Trends Biochem. Sci 2001;26:209–210. [PubMed: 11295538]
5. Gardner PR. J. Inorg. Biochem 2005;99:247–266. [PubMed: 15598505]
6. (a) Gardner PR, Gardner AM, Martin LA, Salzman AL. Proc. Natl. Acad. Sci. U. S. A 1998;95:10378–10383. [PubMed: 9724711] (b) Gardner AM, Gardner PR. J. Biol. Chem 2002;277:8166–8171. [PubMed: 11751864]
7. Herold S. FEBS Lett 1999;443:81–84. [PubMed: 9928957]
8. Herold S, Exner M, Nauser T. Biochemistry 2001;40:3385–3395. [PubMed: 11258960]
9. Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, Lemon DD. Free Radic. Biol. Med 2004;36:685–697. [PubMed: 14990349]
10. Spiro, TG. Resonance Raman Spectroscopy of Metalloporphyrins. New York: John Wiley & Sons; 1988.
11. Feis A, Marzocchi MP, Paoli M, Smulevich G. Biochemistry 1994;33:4577–4583. [PubMed: 8161513]
12. (a) Tsai J-HM, Harrison JG, Martin JC, Hamilton TP, van der Woerd M, Jablonsky MJ, Beckman JS. J. Am. Chem. Soc 1994;116:4115–4116. (b) Lo W-J, Lee Y-P, Tsai J-HM, Tsai H-H, Hamilton TP, Harrison JG, Beckman JS. J. Chem. Phys 1995;103:4026–44034.
13. Gulyan GM, Kurtikyan TS, Ford PC. Inorg. Chem 2008;47:787–789. [PubMed: 18163616]
14. Blomberg LM, Blomberg MRA, Siegbahn PEM. J. Biol. Inorg. Chem 2004;9:923–935. [PubMed: 15452775]
15. (a) Merryweather J, Summers F, Vitello LB, Erman JE. Arch. Biochem. Biophys 1998;358:359–368. [PubMed: 9784251] (b) Wanat A, Gdula-Argasinska J, Rutkowska-Zbik D, Witko M, Stochel G, van Eldik R. J. Biol. Inorg. Chem 2002;7:165–176. [PubMed: 11862553]
16. (a) Cherpanov AV, de Vries S. Biochim. Biophys. Acta 2004;1656:1–31. [PubMed: 15136155] (b) Wiertz FGM, Richter O-MH, Cherepanov AV, MacMillan F, Ludwig B, de Vries S. FEBS Lett 2004;575:127–130. [PubMed: 15388346] (c) Lu S, Wiertz FGM, de Vries S, Moënne-Loccoz P. J. Raman Spectrosc 2005;36:359–362.

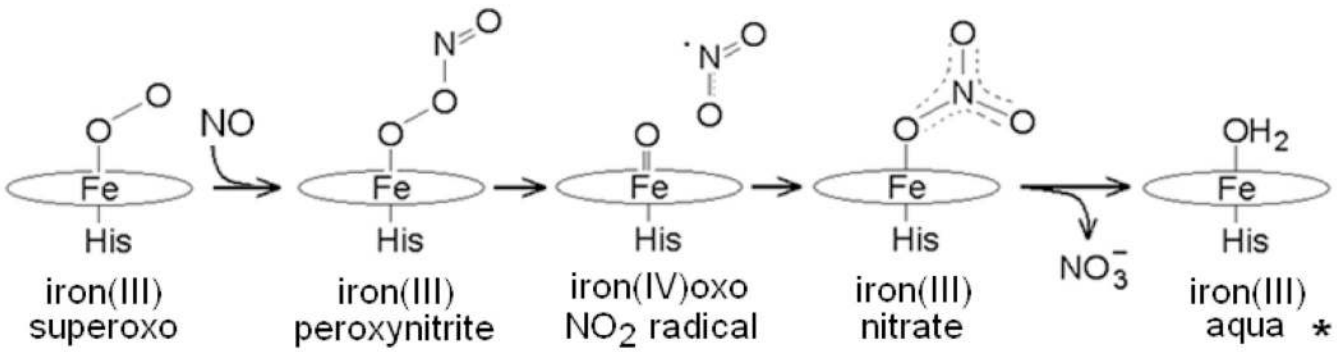

Figure 1.
Proposed NO dioxygenation by oxyheme (* at alkaline pH, the iron(III)aqua complex of Myoglobin is replaced by an iron(III)hydroxo complex, which adopts a low-spin configuration at cryogenic temperatures).

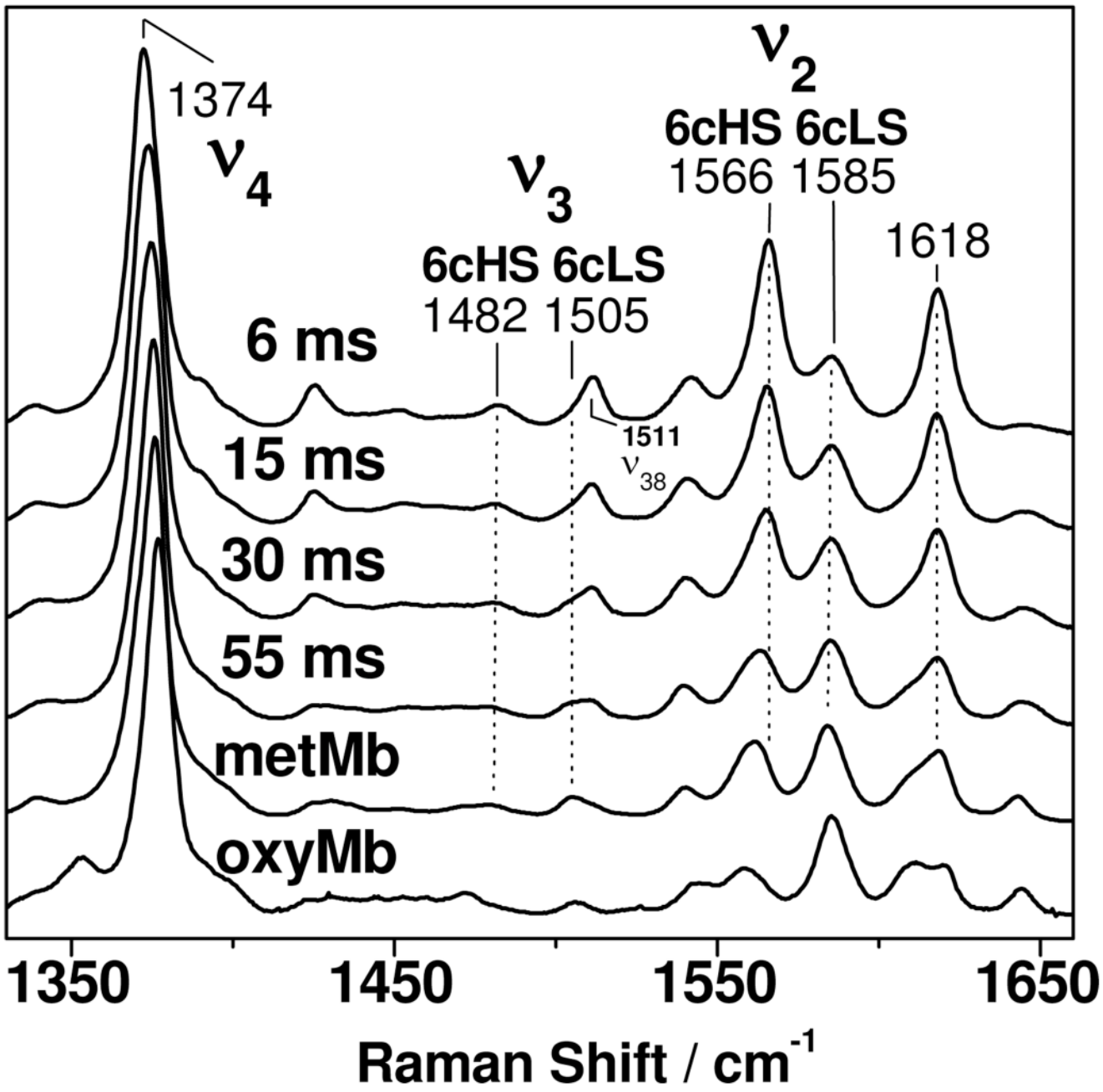

Figure 2.
High-frequency RR spectra obtained with 413-nm excitation at 105 K on RFQ samples (reaction times: 6 ms, 15 ms, 30 ms, and 55 ms) of Mb-O_2 + NO at pH 9.5. Also shown are the RR spectra of the product and starting material at pH 9.5, metMb and oxyMb, respectively.

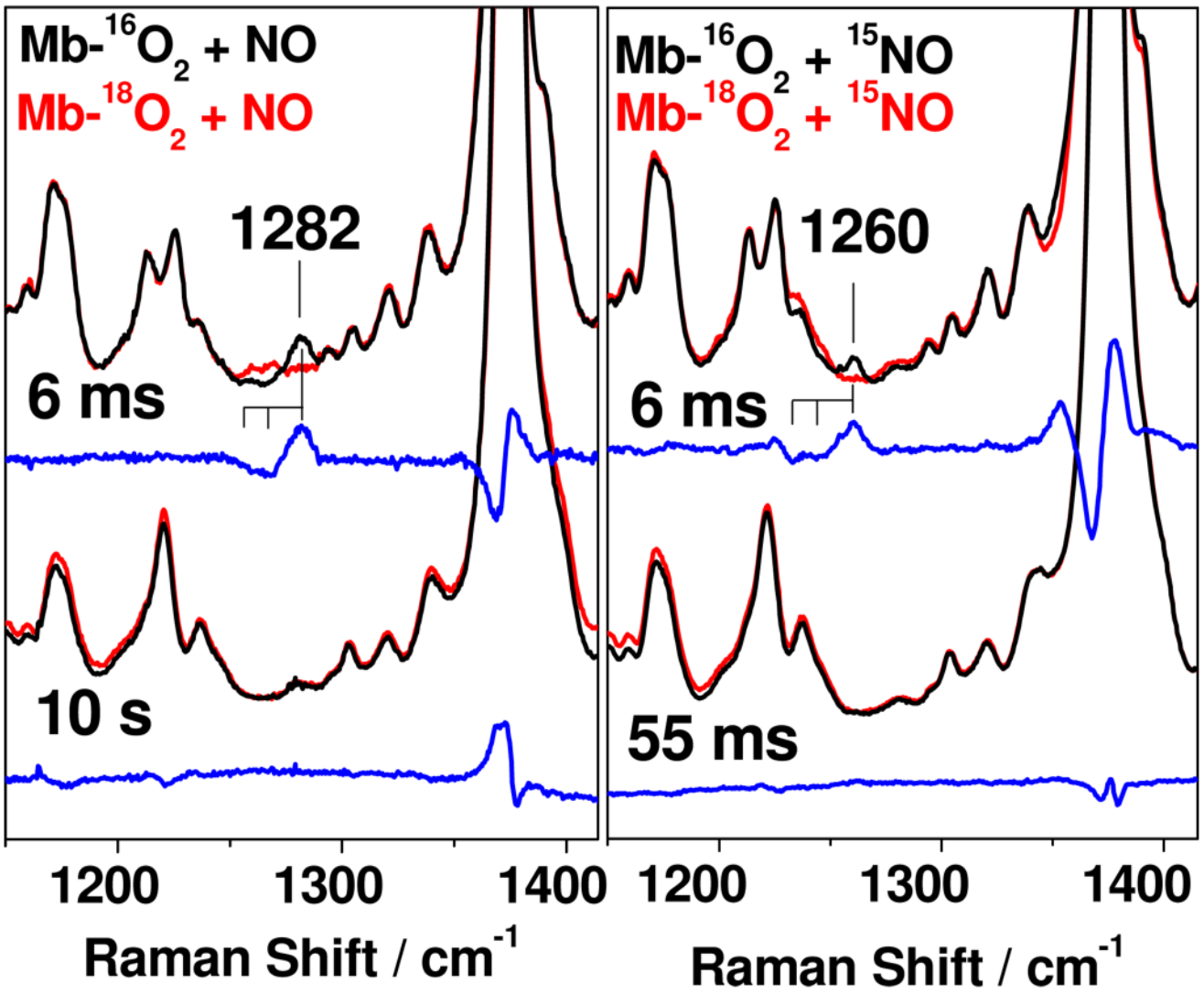

Figure 3.

Mid-frequency RR spectra of RFQ samples for the reaction of ^{14}NO (left) and ^{15}NO (right) with Mb-$^{16}O_2$ (black) versus Mb-$^{18}O_2$ (red). Difference spectra are shown in blue; the differential signals occurring at the intense ν_4 mode (1374 cm^{-1}) vary in independent experiments and represent less than 10% of the integrated ν_4 area.