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Resonance Raman detection of the heme Fe(II)-NO/2-nitrovinyl species in myoglobin

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ABSTRACT

The six-coordinate heme Fe(II)-NO/2-nitrovinyl species in myoglobin has been detected and characterized by resonance Raman spectroscopy. The Fe(II)-^{14}NO and ^{15}N-O stretching frequencies of the ferrous heme nitrosyl/2-nitrovinyl species are detected at 560 and 1587 cm^{-1}, frequencies that are similar to those observed in the Mb heme Fe(II)-NO species. For the 2-nitrovinyl (C_a=C_bNO_2) moiety, which is formed upon H-abstraction from the -C_bH_2 group, the $\nu_s(NO_2)$ is observed at 1322 cm^{-1}, the $\nu_{as}(NO_2)$ at 1516 cm^{-1} and the $\nu(C_a{=}C_b^{14}NO_2)$/ $\nu(C_a{=}C_b^{15}NO_2)$ at 1623/1615 cm^{-1}. The frequencies of the 2-nitrovinyl are largely unaffected by NO_2^-/NO binding to the heme Fe(II)/(III). The properties of the six-coordinate heme Fe(II)-NO/2-nitrovinyl species are compared to those of six-coordinate heme Fe(II)-NO and the five-coordinate heme Fe(II)-NO species isolated from meat products.

1. Introduction

Nitrate and nitrite are the most effective curing agents to prevent bacterial contamination of meat products, although concerns regarding *N*-nitrosamine formation have been raised [1–5]. Nitrate can be reduced to nitrite in raw meat products by microorganisms, whereas in the stomach nitrite can form carcinogenic *N*-nitrosamines [1–5]. Nitrite is bound to proteins in meat and part of it is bound to myoglobin (Mb) leading to the formation of the Mb-NO complex upon nitrite reduction to NO [1]. The nitrite chemistry of Mb has provided a model system for investigating the nitrosation/nitrosylation products formed during meat curing [1–5]. Cured meat differs in composition and eating quality from process or fresh meat and can be combined with other processes including heating, smoking and drying. The flavor of cured meat has been attributed to the presence of nitrite, the oxidation of lipids and due to the proteins components. Cured color develops in the presence of reducing agents and NO, which is an important decomposition product formed by the reduction of nitrate and nitrite and also involved directly into curing and color fixation reaction with Mb in muscle tissue. A number of factors are important in the formation and stability of cured meat color, which is important in products such as bacon, ham, and sausages [1–5]. Although excess nitrite promotes formation of green discoloration by degradation of the heme portion of the molecule it is necessary for supplying the required NO.

Resonance Raman (RR) spectroscopy is a reliable structure sensitive technique, which in conjunction with the use of isotopes can be applied to elucidate the biological activity of heme proteins that activate small molecules [6–10]. In our previous work we have applied this approach to investigate the interactions of nitrite with Mb to address the nitrite reductase activity of Mb, which cannot be elucidates without the determination of the structures of the heme-bound substrate [6–9]. The information from resonance Raman spectroscopy revealed the pH dependent structural changes that occurred in the nitrito heme Fe(III)–*O*–N=O/2-nitrovinyl species [6]. The vibrational characterization of the reversible low to high spin transition of the Fe(III)–*O*–N=O/2-nitrovinyl species provided evidence that the spin-change was trigged by the increase of the proximal Fe-His93 bond length [7,8]. The frequencies of the O– and *N*– sensitive bands were essentially unaffected by the spin change in the Fe(III)–*O*–N=O/2-nitrovinyl species [7,8]. It was thus suggested that the “greening” process in the reaction of metMb with NO_2^- occurs through the formation of the Fe–*O*–N=O/2-nitrovinyl species, which may be present in either the high or low-spin state.

In the present work we have extended our resonance Raman approach to investigate the properties of the Mb heme Fe(II)–NO/2-nitrovinyl species, which is formed subsequent to the reduction of the heme Fe(III)–*O*–N=O/2-nitrovinyl species. The ν(Fe(II)

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$-^{14}NO$) and the $\nu(^{15}NO)$ are detected at 560 and 1587 cm^{-1}, respectively, and are very similar with those observed in the Mb heme Fe(II)–NO species [11]. The $\nu(^{14}NO)$ is not self-evident, because it is obscured by the peak/trough of the 2-nitrovinyl $C_a{=}C_b^{14}NO_2/C_a{=}C_b^{15}NO_2$ stretch at 1623/1615 cm^{-1}. The frequency shifts of the 2-nitrovinyl $C_a{=}C_bNO_2$ vibrations upon reduction and binding of NO to the heme is at most 2-3 cm^{-1} shifted compared to those of the heme Fe(III)–*O*–N=O/2-nitrovinyl species. With the characterization of the Mb heme Fe(II)–NO/2-nitrovinyl species mechanistic issues regarding the formation and properties of the Mb green pigment are addressed.

2. Materials and methods

Analytical grade chemicals, isotopically labeled $Na^{15}NO_2$ (98% ^{15}N, 95% CP) and lyophilized powder of equine skeletal muscle myoglobin (95–100%) were purchased from Sigma Aldrich. The metmyoglobin solutions were prepared by dissolving Mb powder in 100 mM of sodium phosphate at pH 6.0. The Mb samples (50 μM;) were incubated with 50 mM sodium nitrite for 5 days. Reduction of the sample was performed under anaerobic conditions using sodium dithionite as the reductant.

The resonance Raman experiments were performed with a 640 mm focal length Czerny-Turner spectrograph (Horiba, T64000 system operated in single stage) equipped with 1800 g/mm holographic grating and a Horiba Symphony BIUV1024x256 CCD detector, as described previously [6–8]. Briefly, the protein samples were transferred under anaerobic conditions in a quartz tube that was rotated to diminish local heating, and the scattered photons were collected in a 90° geometry. Rayleigh scattering was rejected by a Semrock StopLine 405 nm single-notch filter in the 405 nm excitation experiments, while for the 441.6 nm excitation experiments a Semrock 442 nm long-pass edge filter was used. The total accumulation time for each spectrum was 15–40 min and the power incident on the sample was 4 mW for both the 405 nm and 441.6 nm excitation experiments. Spectra processing and analysis were performed with Origin software and toluene was used for calibration of the Raman shifts.

3. Results and discussion

The resonance Raman spectra of heme proteins contain marker bands in the high frequency region that provide information on the oxidation (ν_4), spin (ν_2, ν_{10}) and coordination (ν_3) state of the heme Fe [6]. Fig. 1A depicts the high-frequency 441 nm excitation RR spectra of Mb incubated in the presence of $^{14}N^{16}O_2^-$ (*trace a*) and $^{15}N^{16}O_2^-$ (trace b) at pH 6 and the corresponding spectra subsequent to addition of dithionite in traces c and d. The high frequency RR bands upon binding of NO_2^- to metMb forming the 450 nm species (green pigment) are in agreement with those previously reported for the formation of a high-spin six coordinate heme Fe(III)–*O*–N=O/2-nitrovinyl species [7,8]. Upon addition of dithionite the shift of ν_4, ν_3 and ν_{10} to 1375 cm^{-1}, 1502 cm^{-1} and 1636 cm^{-1}, respectively, indicate the formation of a new ferrous six-coordinate low-spin species. The difference spectrum a-b ($^{14}N^{16}O_2^-$ *minus* $^{15}N^{16}O_2$) shown in Fig. 1B (with enlarged view in inset B1) is in full agreement with those reported in previous work for the 2-nitrovinyl moiety ($C_a{=}C_bNO_2$) that is formed upon H-abstraction from the $-C_bH_2$ group, with the $\nu_s(NO_2)$ frequency at 1324 cm^{-1}, the $\nu_{as}(NO_2)$ frequency at 1518 cm^{-1} and the peak/trough at 1626/1618 cm^{-1} assigned to the 2-nitrovinyl $\nu(C_a{=}C_b^{14}NO_2)/\nu(C_a{=}C_b^{15}NO_2)$ [7,8]. The difference c-d spectrum ($^{14}N^{16}O_2^-$ *minus* $^{15}N^{16}O_2$) shows small frequency shifts in the peaks/troughs of the $\nu_{as}(^{14}NO_2)/\nu_{as}(^{15}NO_2)$ stretching frequency which is observed at 1516/1482 cm^{-1}, whereas the $\nu(C_a{=}C_b^{14}NO_2)/\nu(C_a{=}C_b^{15}NO_2)$ that is observed at 1623/1615 cm^{-1} shows a 3 cm^{-1} downshift. A 2 cm^{-1} downshift is also observed for the $\nu_s(NO_2)$ at 1322 cm^{-1}. In addition, a negative peak at 1587 cm^{-1} is present in the c-d spectra ($^{14}N^{16}O_2^-$ *minus* $^{15}N^{16}O_2$), as shown in the enlarged view of inset B1. In the low frequency region of the Raman spectrum shown in the inset B2 a peak/trough at 560/535 cm^{-1} is present, which is very similar to that assigned to the ν(Fe(II)–NO) of Mb-NO as presented in the supplementary material (Fig. S1, resonance Raman spectra of the Mb(II)NO species) and in agreement with previous work [11].

Furthermore, the mode we have observed as a negative peak at 1587 cm^{-1} has been assigned to the $\nu(^{15}NO)$ of the Mb-NO species [11] and confirmed in our study (Fig. S1). The $\nu(^{14}NO)$ is obscured by the peak/trough of the 2-nitrovinyl $C_a{=}C_b^{14}NO_2/C_a{=}C_b^{15}NO_2$ stretch at 1623/1615 cm^{-1}. The frequencies and the isotope shifts are in agreement with those previously reported [11]. It should be noted that upon coordination of NO_2^- to the vinyl group a significant change in ν_{11} (C_β-C_β stretch) is induced. The ν_{11} is observed at 1560 cm^{-1} in the Mb-NO species shown in Fig. S1, while the coordination of the NO_2^- on the vinyl induces a 10 cm^{-1} decrease in the frequency of ν_{11} and it is observed at 1551 cm^{-1}. Therefore, in addition to the isotope-sensitive NO_2^- vibrations the heme macrocycle C_β-C_β vibration, ν_{11}, can be used as a marker mode for the NO_2^- coordination to the 2-vinyl in the heme Fe(II)–NO/ 2-$C_a{=}C_bNO_2$ species. In the case of the isotopic frequency shifts for the $^{14}N^{16}O$, a 29 cm^{-1} is expected for the diatomic NO molecule. Therefore, a mode near 1616 cm^{-1}, which is obscured by the peak of the $\nu(C_a{=}C_b^{14}NO_2)$ at 1626 cm^{-1}, can be assigned to the $\nu(^{14}NO)$ of the bound to the heme Fe(II)–NO. When the bent heme Fe(II)–NO interacts with the $C_a{=}C_bNO_2$ as well as the heme macrocycle outer C atoms, vibrational mixing can occur perturbing the $\nu_2(C_\beta$-$C_\beta)$ at 1586 cm^{-1} and the 2-nitrovinyl at 1626 cm^{-1}. The vibrational mixing would be larger as the ν(NO) approaches the frequency of the $C_a{=}C_b^{14}NO_2$ at 1626 cm^{-1} resulting in intensity sharing and larger shifts from the intrinsic frequency. The above-mentioned assignment of the vibrational modes of the heme Fe(II)–NO/2-$C_a{=}C_bNO_2$ species was confirmed by the 405 nm excitation spectra shown in Fig. 2.

The ligand binding in Mb has been established by a variety of spectroscopic techniques and the role of specific residues has been studied in detail [12–16]. Nitric oxide (NO) can act as a reductant forming nitrosonium (NO^+) or as an oxidant forming the nitroxyl anion (NO^-). The formation of Fe(II)–NO from Fe(III) and nitrite using reducing agents such as dithionite or ascorbate is a common approach [11,17]. Nitrate can substitute nitrite as a curing agent to prevent bacterial contamination, but it is then reduced to NO_2^- either chemically or by microbial methods. Nitrous acid is reduced by ascorbate to yield NO and reduction of Fe(III) takes place by either reductive nitrosylation with NO under aerobic or anaerobic conditions and ascorbate reduces both the heme Fe(III) and nitrite or direct reaction with ascorbate [17,18]. Nitric oxide binds to the heme Fe(II) with higher affinity than O_2, and also to heme Fe(III), but with lower affinity than that of the heme Fe(II) ($K_d = 1x10^{-14}$ [M^{-1}]) [11,17,18]. The distal His64 is a key player in the ligand binding properties the heme Fe and in this case has been involved in providing hydrogen bonding to the NO-bound to the heme Fe(II), swinging out into the solvent when it becomes protonated at acidic pH [11,19]. The reactivity of the bound NO is regulated by the strength of the proximal Fe-His93 bond, which is cleaved at acidic pH [11,19]. At neutral pH, the frequency of the ν(Fe(II)-His) is located at 220 cm^{-1} indicating that the Fe-His93 bond is of medium strength in deoxyMb and upon NO coordination to the heme Fe(II) a stable six-coordinated nitrosylheme adduct is formed [11]. The replacement of H64 and V68 in the distal heme site changes the affinity of ligand binding without influencing the strength of the Fe-His93 bond, although it has been suggested that communication