Substrate Binding to a Nitrite Reductase Induces a Spin Transition

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The multiheme enzyme nitrite reductase catalyzes a 6-electron reduction of nitrite to ammonia. The reaction is initiated by substrate binding to the free axial position of the high spin penta-coordinated heme active site. The spin configuration of the resulting complex is crucial for discrimination between the heterolytic vs homolytic character of the cleavage of the N−O bond and, therefore, subsequent steps of the catalytic cycle. Here, we report the first experimental evidence, based on resonance Raman spectroscopy, that nitrite binding to the enzyme from *D. vulgaris* induces a transition from the high spin to the low spin configuration in the catalytic heme, thereby favoring the heterolytic route.

Introduction

Dissimilatory cytochrome *c* nitrite reductases (ccNiR or NrfA gene product) are heme proteins that catalyze the last step in the anaerobic respiration process of nitrite ammonification.[1] This enzyme is expressed in *D. vulgaris*, although the bacterium cannot grow by nitrite ammonification. Instead, the reductase helps this organism to overcome inhibition by nitrite produced by other bacteria in their habitat.[2–4] The nitrite reductase from *D. vulgaris* (NrfHA) is a multiheme enzyme, typically purified as a stable complex of four soluble pentahemic (NrfA) and two transmembranar tetrahemic (NrfH) subunits.[3] The latter receive electrons from the membrane quinone pool and transfer them to the NrfA, where the reduction of nitrite takes place.[5] The active site situated in the NrfA subunit (one per monomer) is a penta-coordinated high spin (5cHS) heme with a lysine residue as the axial ligand at the proximal side.[6] This is an exceptionally rare example of histidine replacement by lysine within the Cys−X_1−X_2−Cys−His binding motif for *c*-type hemes. The presence of a highly conserved positively charged arginine residue in the distal pocket is believed to be determinant for substrate binding to the active site.[3,6–11] The NrfA houses four more low spin (LS) hemes *c*, that take part in electron transfer (ET) from NrfH to the nitrite reduction site. They are all positioned within distances that allow direct electron tunnelling between them, as shown by the available X-ray structures.[3,6–12] Nitrite reductases are also capable of 6-electron reduction of sulfite to sulphide,[13] although the specific activity is several orders of magnitude lower than that for nitrite, and varies substantially between the enzymes from different organisms.[11] The membrane anchoring NrfH subunit houses three bis-His coordinated LS hemes *c* and one 5cHS heme *c* which carries an unusual axial methionine ligand and interacts with menaquinone.[3]

The catalytic reaction starts with binding of nitrite at the distal position of the Fe(II) active site via the N-atom, as inferred from the crystal structure of the complex of the ferric enzymes with the substrate. Density functional calculations (DFT) suggested a spin state transition, from HS to LS, of the heme group in the active site upon substrate binding, although no experimental evidence has been reported. Combined experimental and theoretical studies indicate that the spin state of the initial enzyme/substrate complex is determinant for the reaction mechanism, as it governs the initial cleavage of the N−O bond.[10,14–17]

Here, we present a resonance Raman (RR) spectroscopic study of the NrfHA from *D. vulgaris*. Specifically, we take advantage of the sensitivity of the method toward the coordination and spin configuration of heme proteins[18–24] to characterize the initial complex of the enzyme with nitrite. The results reveal the first experimental evidence for substrate-induced spin transition in nitrite reductases.

Materials and Methods

The NrfHA nitrite reductase from *D. vulgaris* was purified as previously described.[2,3]

RR measurements were performed with a confocal microscope coupled to a single stage spectrograph (Jobin Yvon XY-800) equipped with 1800 lines/mm grating and a liquid-nitrogen-cooled back-illuminated CCD detector. Samples (25−40 μM concentration) were placed in a quartz rotating cell and excited with the 413 nm line from a Kr^+ laser (Coherent Innova 302). Prior to the final choice of experimental conditions, the effect of laser power on the RR spectra of the ferric sample was tested. No signs of photoreduction were observed in the 0.5−5 mW range at RT. All RR spectra of oxidized (as purified) and $NaBH_4$-reduced NrfHA were measured with 1 mW laser power and 40 s accumulation time at room temperature. The reported RR spectra represent an average of 4−10 individual spectra.

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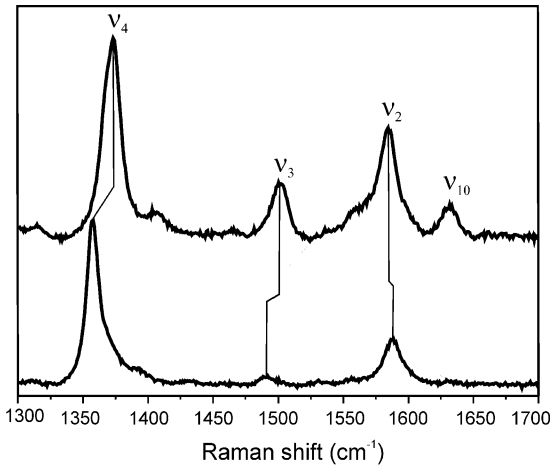

Figure 1. Resonance Raman spectra of ferric (top) and ferrous (bottom) NrfHA (30 μM in 50 mM Tris−HCl buffer, pH 7.6). Spectra were obtained with 413 nm excitation, 40 s accumulation time, and 1 mW laser power.

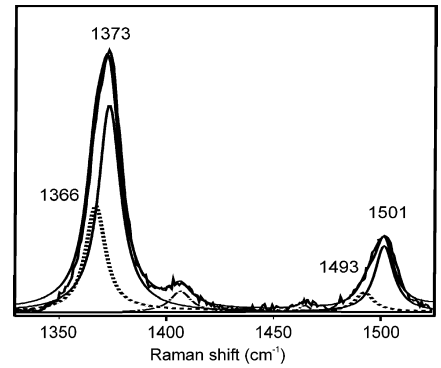

Figure 2. Experimental, component, and simulated RR spectra of ferric NrfHA. The ν_4 and ν_3 modes of the HS (dotted line) species at 1366 and 1493 cm^{-1} and of the LS (solid line) species at 1373 and 1501 cm^{-1}, respectively, were simulated with $\Delta\nu_4$ and $\Delta\nu_3$ of 13.5 and 8.7 cm^{-1} for the HS and 14 and 9.9 cm^{-1} for the LS populations. Experimental conditions as in Figure 1.

Substrate binding was followed by measuring the RR spectra of the oxidized NrfHA upon subsequent additions of four 10 μL aliquots of 10 mM, $Na^{14}NO_2$, $Na^{15}NO_2$, or Na_2SO_3. After polynomial background subtraction, the high frequency region of the RR spectra was subjected to a band fitting analysis.[22,23,25] Homemade software was used to simulate the experimental spectra as a convolution of a minimum number of Lorentzian bands that allows for a good reproduction of the experimental data, using band positions, widths, and intensities as adjustable parameters. Spectral contributions of the HS and LS species were determined from the integrated areas of the ν_3 and ν_4 band components.

Results and Discussion

Spectroscopic Characterization of the Resting Enzyme. RR spectra of heme proteins recorded under Soret-band excitation are, in the high frequency region, dominated by the vibrational modes ν_4, ν_3, ν_2, and ν_{10} that are sensitive spectral markers of the redox, spin, and coordination state of the heme iron.[18–21,26] The transition from a six-coordinated LS (6cLS) heme to a 5cHS heme is associated with an increase of the size of the iron ion and reflected by a downshift of the corresponding vibrational modes. Similarly, reduction of the heme iron, while maintaining the spin and coordination state, causes an increased back-donation of electron density into the π^* orbital of the porphyrin of ferrous heme and thus results in a downshift of the mode ν_4. These empirical relationships have been established in a large number of experimental studies of heme proteins and model compounds and thus provide a sound basis for the RR spectroscopic analysis of the heme coordination and electronic configuration of unknown species.[18–23,25,26]

Figure 1 shows the marker band region of the RR spectrum of NrfHA from *D. vulgaris* in the fully oxidized (top trace) and fully reduced (bottom trace) states. A first visual inspection indicates that the bands ν_4, ν_3, and ν_2 appear at positions characteristic for 6cLS ferric (1373, 1501, and 1583 cm^{-1}) and ferrous (1357, 1490, and 1588 cm^{-1}) species.[20–23] The bands, particularly for the ferric form, are broader than usual and asymmetric, deviating from the expected Lorentzian shape.[23] Namely, in the RR spectra of ferric heme proteins with single spin population, natural line widths ($\Delta\nu$) of the ν_4 and ν_3 modes, defined by the lifetime of the state, typically do not exceed 13−15 cm^{-1} for the former and 8−10 cm^{-1} for the latter mode. In the spectra of ferric NrfHA, the observed $\Delta\nu_4$ and $\Delta\nu_3$ are 17 and 12.5 cm^{-1}, respectively, indicating the presence of more than one spin population. Indeed, a careful band fitting analysis shows that in order to reproduce the ν_3 and ν_4 envelopes of the fully oxidized NrfHA a minimum of two well separated and physically meaningful bands is required in each case (Figure 2). This finding reflects a coexistence of LS and HS species in the RR spectra, originating from the ET and catalytic hemes, respectively.[3] For the LS species, the ν_4 and ν_3 bands are found at 1373 and 1501 cm^{-1}, respectively, with an intensity ratio of 3.3:1. The corresponding modes of the HS species appear at 1366 and 1493 cm^{-1}, respectively, with an intensity ratio of 5.1:1. Both the positions and relative intensities of the bands are consistent with RR spectra of LS and HS hemes reported in the literature.[19–23] The ν_3 bands originating from HS and LS hemes typically appear well separated in the spectra of ferrous proteins;[19] however, this region is completely dominated by the LS hemes in the spectrum of the fully reduced, large NrfHA complex (Figure 1, bottom trace), impeding a reliable spectral analysis. Similarly, no attempt was made to distinguish between the two types of 5cHS ferric hemes, i.e., Met-Fe(III) and Lys-Fe(III), present in NrfH and NrfA, respectively, since their vibrational modes are expected to be at very similar frequencies. Thus, although a distinction between the individual HS hemes was not possible, we could evaluate the RR spectra in terms of HS and LS contributions for the resting (fully oxidized) enzyme. In this case, the relative spectral HS:LS ratio was determined to be 1:2 on the basis of the integrated areas of the ν_3 and ν_4 band components (Figure 2). Taking into account the amount of HS and LS hemes in the protein complex (6 and 22, respectively), this value indicates that, for the 413 nm excitation used in the present experiments, the RR cross sections of the HS modes are larger than those of the LS modes by a factor of ca. 1.8.

Substrate Binding. The six-electron reduction of nitrite to ammonia by nitrite reductases has been proposed to be initiated by the binding of the substrate to the free axial positions of the HS Lys-Fe(II) hemes.[10] Due to the high specific activity of NrfHA, binding of nitrite to the ferrous active site leads to immediate turnover, hampering the characterization of the initial enzyme/substrate complex.[10] Therefore, we have studied the interaction of the resting (fully oxidized) NrfHA with nitrite, as a model for the initial step of the catalytic reaction.

RR spectra were measured after successive additions of nitrite aliquots to the oxidized enzyme, until achieving a final 200-

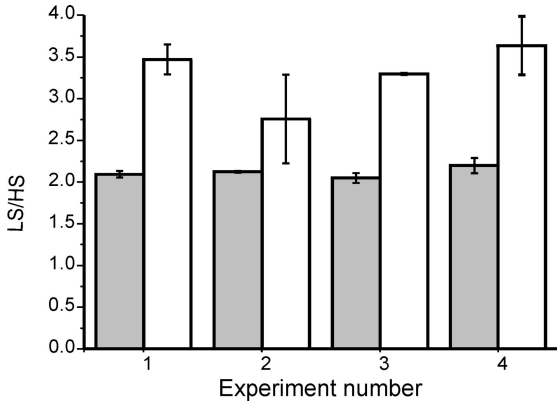

Figure 3. Reproducibility of the spin transition determination. Ratio of the spectral contributions of the LS vs the HS species in the ferric NrfHA, in the absence (gray) and presence (white) of nitrite, as determined from the integrated area of the (ν_4 and ν_3) band components in four independent experiments; the error bars represent deviation from the average value of the LS/HS ratio determined in the absence/presence of the substrate.

fold excess. Even spectra recorded with the highest nitrite excess look qualitatively similar to the RR spectrum of ferric NrfHA in the absence of substrate. Moreover, the spectra can be perfectly simulated using the component bands determined for the substrate free enzyme.

A distinct feature, however, is that the relative spectral contribution of the HS species to the ν_3 and ν_4 envelopes drops by 40% in the presence of nitrite, from a LS:HS ratio of 2 in the absence of nitrite to a value of 3.35 in the substrate−enzyme complex. The change of the LS:HS ratio upon nitrite addition is reproducible and reaches a maximum value of 3.35 for a 50-fold excess of nitrite, Figure 3. These results are interpreted in terms of binding of nitrite to the free axial position of some of the HS hemes, thereby inducing the conversion from the 5cHS to the 6cLS state.

Nitrite can bind to the heme either via nitrogen or via oxygen atom, as demonstrated for various nitrite reducing enzymes.[8,10] Previous DFT calculations with model systems predicted that binding of nitrite to the ferric HS heme via oxygen is energetically unfavorable and results in a HS ($S = 5/2$) configuration. The energetically favored configuration is established upon binding via the nitrogen atom, and is predicted to lead to a LS ($S = 1/2$) state. The crystal structure of the enzyme/substrate complex from *W. succinogenes* corroborates N-binding of nitrite to the Lys-Fe(III) HS hemes.[10] The NrfHA from *D. vulgaris* is also expected to bind nitrite only to the Lys-Fe(III) HS hemes of the NrfA subunits. Binding to the Met-Fe(III) HS hemes from subunit NrfH is highly unlikely because of the steric hindrance of the distal aspartate residue (Asp89), whose carboxylate group is positioned parallel to the heme plane, as shown by the X-ray structure.[3] Thus, we conclude that in the *D. vulgaris* enzyme nitrite binds to the Lys-Fe(III) HS hemes of the NrfA subunits via the nitrogen atom, and that the binding induces a transition from the HS to the LS configuration. The binding is substoichiometric, as the drop of the HS spectral contribution indicates that only half of the Lys-coordinated heme groups undergo the spin state transition. This is not surprising taking into account that the affinity of the ferric enzyme for nitrite is significantly lower than that of the ferrous form.[10]

No new bands were identified in the low frequency region in spite of careful comparison of the RR spectra of the enzyme obtained in the presence of $^{14}NO_2$ and $^{15}NO_2$. We attribute the absence of Fe-ligand modes to enhancements that are too weak for detection of these modes among the large number of porphyrin bands of 28 heme cofactors present in the NrfHA complex.

The NrfHA from *D. vulgaris* also reduces sulfite to sulfide.[3,13] As in the case of nitrite binding, addition of sulfite to the fully oxidized enzyme does not alter the RR spectral parameters but produces an 18% drop of the relative HS contribution to the ν_3 and ν_4 envelopes (data not shown). Quantitatively, the effect is about one-half of that observed for nitrite binding and close to the error margin of the method. Nevertheless, this finding can be attributed to the lower affinity of the oxidized enzyme for sulfite, which is in agreement with a longer Fe(III)−S bond compared to Fe(III)−N (2.4 Å vs 1.9 Å), observed in the corresponding crystal structures.[11,12]

Conclusions

Binding of nitrite to the active sites of nitrite reductases, i.e., the conserved Lys-coordinated HS hemes, via the N-atom has been shown before by crystallographic data. DFT calculations on model systems predicted that such binding results in a HS to LS transition,[10] but no experimental evidence has been provided to date. The spin configuration of the initial enzyme/substrate complex has profound consequences for the reaction mechanism of nitrite reduction, as it determines the character of the N−O bond cleavage, i.e., homolytic vs heterolytic, and therefore also the subsequent steps of the catalytic cycle.[10,14–17]

The RR data presented in this work provide the first experimental evidence that nitrite binding to the active site of the NrfHA from *D. vulgaris* indeed causes a spin conversion to the LS configuration, thereby favoring the heterolytic cleavage of the N−O bond as the first step of the catalytic reaction. Most likely, sulfite reduction follows the same mechanistic pathway.

It is worth noting that the RR studies were performed on the enzyme in its fully oxidized form, since the fast turnover of the enzyme prevents stationary studies of the active initial (ferrous) complex. This approach, also adopted in previous studies,[10,11] however, does not invalidate the conclusions. Previous experimental and theoretical studies on model systems show that the affinity of nitrite for Fe(II) is higher than that for Fe(III) and, moreover, that the HS to LS transition is energetically more favorable for Fe(II).[10] Thus, the effects observed here by RR spectroscopy of the oxidized enzyme are expected to be even more pronounced for the reduced NrfHA.

The nitrite reductases characterized so far show a high degree of homology and share common three-dimensional structures.[3,6–8,10,11] In all cases, the active sites reveal common features, including (i) a Lys-coordinated HS heme, (ii) aromatic residues that restrict solvent accessibility, thereby modulating the reduction potential of the heme, (iii) a conserved arginine at the distal side that facilitates substrate binding, and (iv) several tyrosine residues capable of quenching radical intermediates along the catalytic cycle. Thus, conclusions derived from the present study on NrfHA from *D. vulgaris* are likely to be relevant for the entire family of nitrite reductases.

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Supporting Information Available: Figure showing resonance Raman and electronic (inset) spectra of the ferric NrfHA complex in the absence (solid lines) and presence (dotted lines)

of an excess of nitrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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