Active Intermediates in Copper Nitrite Reductase Reactions Probed by a Cryotrapping-Electron Paramagnetic Resonance Approach

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Abstract: Redox active metalloenzymes catalyse a range of biochemical processes essential for life. However, due to their complex reaction mechanisms, and often, their poor optical signals, detailed mechanistic understandings of them are limited. Here, we develop a cryoreduction approach coupled to electron paramagnetic resonance measurements to study electron transfer between the copper centers in the copper nitrite reductase (CuNiR) family of enzymes. Unlike alternative methods used to study electron transfer reactions, the cryoreduction approach presented here allows observation of the redox state of both metal centers, a direct read-out of electron transfer, determines the presence of the substrate/product in the active site and shows the importance of protein motion in inter-copper electron transfer catalyzed by CuNiRs. Cryoreduction-EPR is broadly applicable for the study of electron transfer in other redox enzymes and paves the way to explore transient states in multiple redox-center containing proteins (homo and hetero metal ions).

Introduction

Transition metals (e.g. Cu, Mn, Fe and Mo) are ubiquitous in biology and play key roles in redox enzymes that are vital for life and the next generation of biofuels.[1] Due to their complex reaction mechanism, and often, poor optical signals (e.g. the weak absorbance bands from the type-II copper complexes), identifying and studying active intermediates in the reaction cycle of metal containing enzymes is challenging. Methods must be established for deeper mechanistic insights into these enzymes. Here, we develop the use of cryoreduction (with annealing at higher temperatures) in combination with electron paramagnetic resonance (EPR) spectroscopy for monitoring and detecting active intermediates in the electron transfer reactions of complex metalloenzymes.

Cryoreduction-EPR involves the use of ionizing radiation to reduce frozen solutions of protein (e.g. 77 K). Then, by annealing the frozen protein solution to higher temperatures, reaction intermediates are formed and trapped, enabling the capture of transient enzyme species, which if paramagnetic, can be investigated using EPR spectroscopy. Active catalytic intermediates in a number of enzymes containing heme catalytic centers[3–5] have been identified using similar cryoreduction-EPR methods (e.g. in identification of ferrichydroperoxo, ferric-peroxo, ferrous-superoxy radical and compound I in the P450 monoxygenase reaction cycle).[6] DNA radicals have also been investigated using cryoreduction-EPR methods.[6,7] In this study, as a proof-of-principle, cryoreduction-EPR is used to detect substrate binding, product release and the active intermediates when electrons are transferred from the type-I to the type-II copper centers in the copper-containing nitrite reductase (CuNiR) family of enzymes.

CuNiRs catalyze the reduction of soluble nitrite to gaseous nitric oxide, a key step in denitrification and the global nitrogen cycle.[8,9] 
\[ \text{NO}_2^- + 2 \text{H}^+ + 1 \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O} \] (1)

Two-domain CuNiRs were first identified over 40 years ago, and have been the subject of extensive biochemical and biophysical studies.[8,9] In the last decade, a number of partner protein-tethered CuNiRs (three-domain CuNiRs) have been isolated and characterized.[10–12] These three-domain CuNiRs maintain similar structures to their two-domain counterparts, but contain cytochrome c or azurin partner proteins fused at their C- or N-termini.[12,13]

Two-domain CuNiRs (e.g. well-characterized Alcaligenes xylosoxidans CuNiR; AxNiR) and three-domain cytochrome c-fused CuNiRs (e.g. Ralstonia pickettii CuNiRs; RpNiR) are trimeric enzymes.[12,14,15] Within each of the three monomeric chains of CuNiRs are two β-sandwich motifs, which both house an individual copper ion, a type I (T1Cu) and type II (T2Cu) copper. In CuNiRs, two histidine residues, a cysteine and an axial methionine residue coordinate the T1Cu center (Figure 1).[12,14] Three histidine residues and
either a water (H\textsubscript{2}O), nitrite (NO\textsubscript{2}^{-}) or nitric oxide (NO) molecule coordinate the T2Cu (Figure 1). Mechanistic studies show that the T1Cu receives electrons from partner proteins (in two-domain CuNiRs) or the fused cytochrome c/azurin domain (in three-domain CuNiRs).[12,13,16] Electrons then transfer from the reduced T1Cu to the catalytic T2Cu ion in the core CuNiR portion of the protein, where nitrite is converted to nitric oxide.[17–19] Inter-copper electron transfer in CuNiRs occurs via a proton coupled electron transfer (PCET) reaction.[17,18,20] Numerous methods, including laser-flash photolysis,[17] pulsed-radiolysis,[18] single-molecule FRET,[21] pH-perturbation,[20,22,23] and serial crystallography[24] have been used to study inter-copper electron transfer catalyzed by CuNiRs. Whilst these approaches have provided valuable contributions to our understanding of electron transfer in CuNiRs, they have been restricted by limited optical signal from the T2Cu center. Here, we used a \(^{60}\)Co source of Y-radiation at 77 K to cryolytically reduce the copper centers in two copper-containing nitrite reductases, the two-domain \textit{Ax}NiR and the core portion of the three-domain \textit{Rp}NiR, in the presence and absence of the substrate, nitrite (Figure 1). Using annealing and EPR spectroscopy, simultaneous tracking of T1 and T2Cu redox centers was used to follow and probe inter-copper electron transfer. Initially, we performed studies on “nitrite-free” forms of the well-characterized two-domain \textit{Ax}NiR and the \textit{Rp}NiR core protein for which an X-ray crystal structure and solution properties have been reported.[15]

Results and Discussion

In Figure 2A, the EPR spectra of the oxidized “nitrite-free” \textit{Ax}NiR and \textit{Rp}NiR core proteins measured at 20 K are shown. These spectra display the presence of the overlapping, four-line, parallel hyperfine features, arising from both the T1Cu and T2Cu centers. From experimental and simulated EPR spectra (see Figure S1) collected on the \textit{Rp}NiR core protein, it is observed that the EPR signals at the perpendicular orientation is split into four hyperfine lines, a feature that is due to the strong hyperfine coupling of the \(^{63,65}\)Cu nuclei with the electron spin of T1Cu center. This splitting is absent in the “nitrite free” \textit{Ax}NiR sample, suggesting subtle differences in the electronic structures of the T1Cu centers present in both of these CuNiR proteins (Figure S1 in the Supporting Information).[25] After irradiation of frozen \textit{Ax}NiR and \textit{Rp}NiR proteins at 77 K with Y-rays from a \(^{60}\)Co source, a reduction in the T1Cu EPR signal is observed (approx. 30% and 50% for \textit{Ax}NiR and the \textit{Rp}NiR core protein, respec-
In our earlier studies, we reported that T1 to T2Cu electron transfer was inhibited in the RpNiR core protein in absence of nitrite.[15] This hypothesis was based on recorded mid-point potentials and a laser flash photolysis assay, which was used to monitor changes in the UV/Vis active T1Cu site when laser pulses were used to rapidly inject the protein with electrons. Using the EPR approach presented here to probe the T2Cu site, we have been able to observe an additional previously uncharacterized T2Cu species, T2Cu[2], present in the RpNiR core protein, which is formed upon reduction of the T1Cu and can facilitate inter-copper electron transfer. We must note that in our cryoreduction-EPR experiments performed on the RpNiR core protein, the percentage signal change attributed to the reduction of the T1Cu site during annealing is far lower than that seen in the AxNiR sample (approx. 15% in RpNiR core and 40% in the AxNiR sample). We attribute this to a larger percentage of T1Cu reduced in the RpNiR core protein during the initial cryolytic reduction process (approx. 30% and 50% for AxNiR and the RpNiR core protein, respectively; Figure 2 and Figure S2,S3), a result that is due to different amounts of exposed Co-Y irradiation on the samples (22 kGy in AxNiR and 50 kGy in RpNiR core), and also plausibly, a result of redox potential differences for the T1Cu centers in the different constructs (+ 255 mV in AxNiR,[16] and + 331 mV in the RpNiR core).[15]

We also performed cryolytic reduction-EPR measurements on “nitrite-bound” forms of AxNiR and the RpNiR core proteins. Continuous wave EPR spectra of “nitrite-bound” AxNiR and the RpNiR core proteins are shown in Figures 3A and C, respectively. As expected, in the AxNiR protein sample containing nitrite, the T2Cu, and not the T1Cu center is altered (A(63,65)NiR; T2; ≈ 370 MHz with g = 2.290; AxNiR). This is indicative of the nitrite being bound to the catalytic T2Cu site. For the “nitrite-bound” RpNiR core protein, there are subtle changes in the T2Cu hyperfine features. Based on our new EPR spectral simulations (Figure S7), approx. 20% of the T2Cu hyperfine features have shifted from a “nitrite-free” to a “nitrite-bound” state when the oxidized RpNiR core was incubated with 5 mM nitrite. No additional changes in the hyperfine features of the T2Cu center were observed upon the addition of supplementary nitrite, suggesting that in an oxidized state only a fraction of the RpNiR core population can accept nitrite. Previous studies have shown that a conserved tyrosine residue, present on a cyt c linking region, blocks nitrite from binding to the T2Cu site of the oxidized full-length RpNiR protein.[33] This tyrosine residue is present in the RpNiR core protein, but occupies an alternative state in the X-ray structure, allowing nitrite to bind.[31] We hypothesize that in solution, this linker and tyrosine residue may occupy multiple conformations, both blocking and allowing access of the nitrite substrate to the T2Cu center.

Temperature dependent changes in the oxidized T1 and T2Cu EPR signals (Cu(I)) of “nitrite-bound”, cryolytically reduced AxNiR are presented in Figure 3B, Figure S2 (absolute EPR signal) and S8 (relative EPR signal). Like the “nitrite-free” samples, and indicative of the involvement of protein motions in inter-copper electron transfer of CuNiRs, there is little or no electron transfer below the “glass-
This would lead to the formation of either $\text{R}_p$ www.angewandte.org $T_1$ to $T_2$ Cu electron transfer monitored in “nitrite-bound” $\text{R}_p$ with strong hyperfine coupling to the $A$ $T$ $A$ $T_3$ $A$ $R_p$ and end-on bound Cu the proton coupled electron transfer to the $=\text{}$ for $A$ $R_p$ $2020$ m $\text{R}_p$ $A$ $Ax$ Rhodobacter sphaeroides signal increases. However, between $210–270$ K, a decrease in the oxidized $T_1$ Cu decreases, while the $T_2$ Cu signal increases. However, between $210–270$ K, a decrease in the oxidized $T_2$ Cu signal and an increase in the population of oxidized $T_1$ Cu are observed. Based on these data, we propose the following mechanism for the $R_p$ NiR core protein. In the oxidized form of the enzyme, 20% of the $T_2$ Cu centers are occupied with nitrite. The reducing conditions available for both $T_1$ Cu and $T_2$ Cu centers lead to the depopulation of the resting state EPR signal of the $T_2$ Cu center. This reduction of the $T_1$ Cu site appears to support binding of the nitrite substrate at the $T_2$ Cu site (shown by a shift in electronic properties and an increase in the $\text{Cu}^{I}$ signal at the $T_2$ Cu site). Following this, electrons transfer from the $T_1$ Cu to the $T_2$ Cu site, which is now fully occupied with nitrite.

In recent work, we proposed that electron delivery to the tethered $R_p$ NiR heme cofactor causes conformational change that is required for nitrite binding and catalysis in 3-domain NiRs. We have also emphasized differences in catalytic mechanism between the full-length and core $R_p$ NiR proteins, which highlight previously unforeseen effects of tethering on enzyme catalysis. Here, we show that in the absence of the heme domain, the $T_1$ Cu of the $R_p$ NiR core protein must be partially or fully reduced to enable nitrite binding and catalysis, which provides new insight into the mechanisms of 2-domain NiRs. For example, it has been shown that values for steady-state Michaelis constants (approx. 10 $\mu$m) during catalysis and dissociation constants (approx. 350 $\mu$m) for oxidized 2-domain NiR–nitrite complexes differ significantly. We have used a 2-domain NiR that (in the oxidized state) is only partially occupied with nitrite prior to performing cryoreduction-EPR. Our studies have shown that $T_1$ Cu reduction stimulates nitrite binding to the catalytic $T_2$ Cu center. This result likely accounts for the disparity in values of the Michaelis constants and NiR–nitrate complex dissociation constants noted above.

**Conclusion**

In summary, the cryoreduction-EPR method can be used to track inter-copper electron transfer in the copper nitrite release of the product, nitric oxide, with the formation of [Cu$^{II}$–OH] redox state at the $T_2$ Cu center. Between $218$ and $238$ K, the signal associated with oxidized nitrite-bound $T_2$ Cu “grows in” once again (Figure 3B, S2 and S8). We suggest that this increase in EPR signal associated with the $T_2$ Cu center is indicative of nitrite binding to the $T_2$ Cu after reduction of nitrite to nitric oxide, and subsequent nitric oxide dissociation.
reductase family of enzymes. Our approach highlights the importance of protein dynamics in inter-copper electron transfer catalyzed by two-domain copper nitrite reductases. To the best of our knowledge, this is the first spectroscopic study that has enabled direct monitoring of electron delivery, nitrite-binding and nitric oxide production at the T2Cu in this family of enzymes. Our work has also enabled simultaneous observation of electron transfer between the T1 and T2 coppers in CuNiRs. The approach we have developed is general and could be used to further understand intra-protein electron transfer in other multi-center copper-containing enzymes that have a minimal UV/Vis optical signal associated with the T2Cu sites such as laccases,[34] peptidylglycine α-amidating monoxygenases[35] and particulate methane monoxygenases.[36]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: copper center · copper nitrite reductase · electron paramagnetic resonance · metalloenzymes · redox enzyme

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