Spectroscopic Characterization of Cryoreduced Metalloenzymes

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Introduction

Enzymes that contain transition metal cofactors are widespread in nature and play pivotal roles in almost every aspect of life. The reactions they catalyze are not only of fundamental importance in biology, but are also industrially relevant. These include some of the most difficult chemical reactions known, such as the reduction of the inert N≡N triple bond, the oxidation of water to yield dioxygen, and the activation and functionalization of inert C-H bonds. The main purpose of our research program is to determine the mechanisms, on a detailed atomic level, by which metalloenzymes catalyze these intriguing reactions. To accomplish this goal, we employ a combination of biochemical, kinetic, and spectroscopic methods to characterize states of the catalytic cycle. In particular, we try to trap and characterize reactive intermediates that are often fleeting (short-lived).

Significant information about such species can be gained from studies on samples that have been exposed to gamma-rays (total dose between 2 to 10 MRad) at near cryogenic temperatures (77 K). This procedure has been termed 'cryoreduction'. It has been demonstrated [1] (and references therein) that this procedure results in the reduction of the metal clusters. Importantly, this reduction is thought to proceed with retention of the geometry of the oxidized cluster, because the molecular motion of the radiolytically reduced metal center is impeded at low temperatures.

This method is extremely valuable for studies of metallocofactors that are in diamagnetic or electron paramagnetic resonance (EPR) -silent forms that do not exhibit an EPR signal. Cryoreduction of EPR-silent cofactors may allow for conversion to one-electron-reduced, EPR-active complexes, which can then be interrogated in detail by paramagnetic methods, such as EPR, ENDOR, ESEEM, and Mössbauer spectroscopies. We apply this methodology to study a variety of metalloenzymes.

Cryoreduction of High-Valent Fe(IV)-Oxo Intermediates

Mononuclear non-heme iron enzymes belong to a mechanistically and functionally diverse enzyme superfamily. In most cases, they couple the activation and four-electron reduction of O_2 to the two-electron oxidation of a co-substrate (e.g. 2-oxo-glutarate) and a two-electron oxidation of the primary substrate, which typically entails the hydroxylation of an aliphatic C-H bond. The operant mechanisms proposed for these enzymes had remained largely untested for decades, because the key reaction intermediates could not be directly observed. To elucidate the mechanism of the enzyme taurine:2-oxo-glutarate dioxygenase (TauD), in which the key hydroxylating intermediate is a Fe(IV)-oxo (ferryl) complex, we carried out cryoreduction experiments. The TauD ferryl intermediate has, unexpectedly, a high-spin (S = 2) ground state configuration that gives rise to unique spectroscopic parameters. Therefore, to obtain definitive evidence that the formal oxidation state of the Fe in this intermediate is indeed +IV, we performed cryoreduction of the purported Fe(IV)-oxo intermediate, which showed its conversion to a high-spin Fe(III) complex, verifying our initial mechanistic hypothesis [2]. We show in Figure 1 the effect of

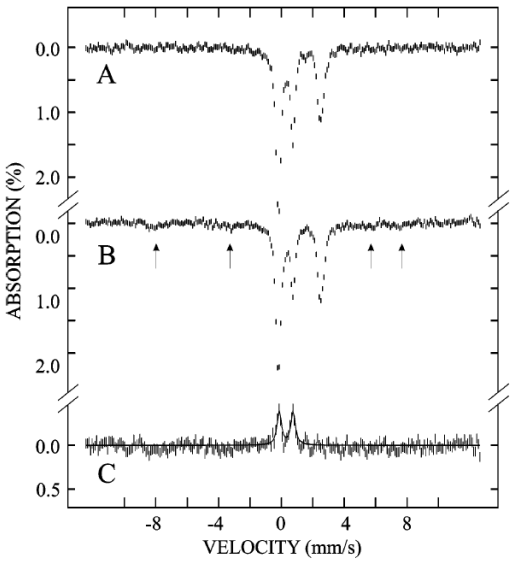

FIGURE 1: Cryoreduction monitored by Mössbauer spectroscopy of a sample containing a ferryl intermediate (adapted from [2]).

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cryoreduction monitored by Mössbauer spectroscopy of a sample containing the ferryl intermediate. Spectra A and B were recorded before and after cryoreduction, respectively. The difference spectrum (C) reveals that the ferryl intermediate (two peaks pointing upwards in the difference spectrum) is reduced to a high-spin Fe(III) complex (broad features indicated by arrows in B. The formation of a high-spin Fe(III) complex is corroborated by EPR spectroscopy on an identical sample (Figure 2). As can be seen, the spectrum after cryoreduction (bottom) exhibits the diagnostic peak at ~1,600 G, which corresponds to the typical isotropic g_{eff} = 4.3 signal associated with high-spin Fe(III). Since then we have adopted this approach and tested this methodology to corroborate the presence of ferryl intermediates in other enzymes, including tyrosine hydroxylase and isopenicillin *N* Synthase (IPNS).

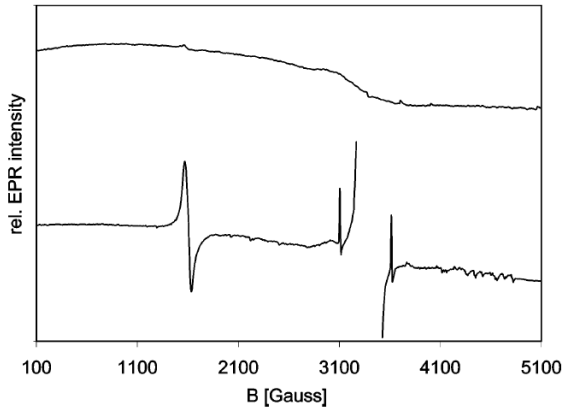

FIGURE 2: The presence of a high-spin Fe(III) complex corroborated by EPR spectroscopy on an identical sample (adapted from [2]).

Cryoreduction of {Fe-NO}^{7} Complexes

The above-mentioned large family of mononuclear non-heme iron enzymes have a proposed common intermediate, a Fe(III)-superoxo complex, which has only very recently been demonstrated experimentally. We used an alternative approach to study this complex by a combination of spectroscopic and computational methods. We used nitric oxide (NO) as a one-electron-deficient analog of the co-substrate O_2 to make the stable {FeNO}^{7} complex and cryoreduced this complex to the corresponding {FeNO}^{8} form. These clusters were studied by a combination of EPR and Mössbauer spectroscopies and the spectroscopic parameters were rationalized computationally. As an example we show the Mössbauer spectra of the {FeNO}^{7} complex before (Figure 3A) and after (Figure 3B) cryoreduction. The features of the {FeNO}^{8} complex are the two intense lines in the difference spectrum (Figure 3C) and the reference spectrum (Figure 3D). We used the two nitrosyl complexes to calibrate the computational methods, and we extrapolated the computations to the elusive Fe(III)-superoxo intermediate [3].

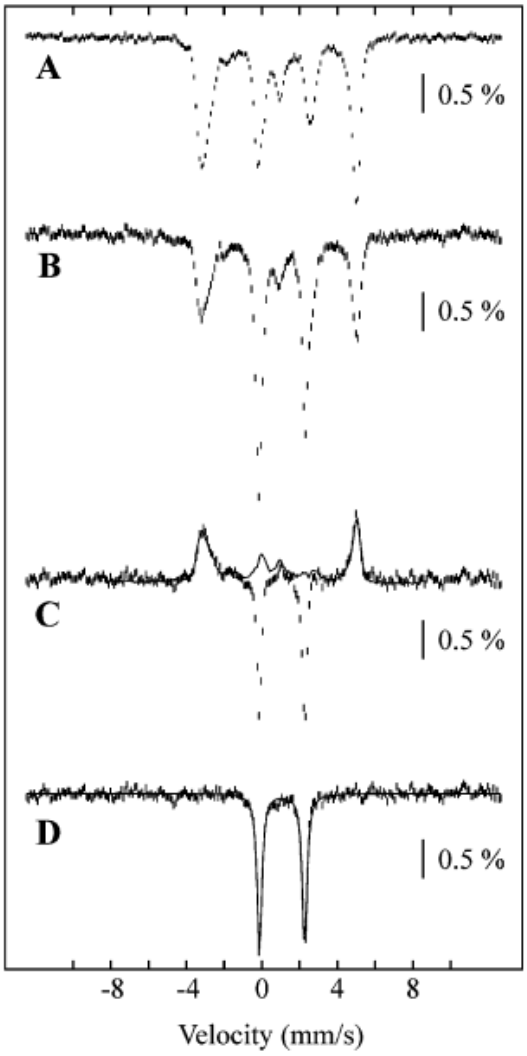

FIGURE 3: Mössbauer spectroscopy of the cryoreduced {FeNO}^{7} complex (adapted from [3]).

Cryoreduction of Mid-Valent Fe_2(III/III)-Peroxo Intermediates

Enzymes that harbor a dinuclear non-heme iron cofactor activate O_2 in a wide variety of oxidation reactions. Their mechanisms are quite diverse, but start with a common first step: the two-electron oxidative addition of O_2 to the reduced Fe_2(II/II) cofactor, to yield a peroxo-Fe_2(III/III) intermediate. All known peroxo-Fe_2(III/III) intermediates exhibit a diamagnetic (S = 0) ground state due to antiferromagnetic coupling between the two high-spin Fe(III) ions. Current evidence suggests that their geometric and electronic structures differ and that these differences may dictate the unique reaction outcomes promoted by the various enzymes. Because of their diamagnetic ground state, these intermediates cannot be probed by EPR-based methods. We have therefore attempted to use cryoreduction to convert them to their one-electron reduced, EPR-active forms. Surprisingly, with a sole

exception, the intermediate from the enzyme aldehyde-deformylating oxygenase (ADO), these intermediates cannot be cryoreduced. ADO is a recently identified dinuclear non-heme iron enzyme that promotes the cleavage of a C_n aldehyde to C_{n-1} hydrocarbon and formate. This reaction is the second of a two-step pathway employed by cyanobacteria in the conversion of fatty acyl substrates to free alkanes. Because the latter can be used as "drop-in" fuels, the pathway and studies of the ADO mechanism have recently been vigorously studied by many groups.

Samples of the alkane producing ADO enzyme that contain a high-yield of the O_2-activated intermediate that initiates the reductive cleavage of O-O and subsequent deformylation of the aldehyde were subjected to cryoreduction experiments. In ADO, conversion of aldehydes to alkanes is catalyzed upon addition of two electrons from the Fe_2(III/III)-peroxide level. The Fe_2(III/III) peroxide carrying intermediate (formally described as a Fe_2(III/III) peroxy-hemiacetal complex) in ADO represents a novel structure for the superfamily of ferritin-like diiron oxygenases/oxidases. We attempted to trap any radical intermediates accumulating upon reduction of the Fe_2(III/III) peroxy-hemiacetal intermediate by cryoreduction experiments. Also we wanted to mimic the cryoreduction effects observed in XAS measurements at the Stanford Synchotron Radation Lightsource (Stanford, CA) and elucidate the chemical nature of the state formed as a result of the intense photoreduciton of the Fe_2(III/III)–peroxyhemiacetal intermediate in the synchrotron.

Mössbauer experiments of those cryoreduced samples indeed showed conversion of this intermediate to a diferric form that has spectroscopic features essentially identical to those of the product-bound complex (after catalytic turnover). In agreement in EPR neither radical intermediates nor the mixed-valent Fe_2(II/III) form were detected, strongly suggesting that the energetics are such that the $Fe_2^{III/III}$-peroxyhemiacetal intermediate gets two electrons reduced, which is in stark contrast with the intermediates in other enzymes of the same structural superfamily (such as methane monooxygenases, ribonucleotide reductases, etc.).

Cryoreduction of $[4Fe\text{-}4S]^{2+}$ Clusters

Metallocenters composed of four Fe ions and four inorganic sulfide ions, which are abbreviated as [4Fe-4S] clusters, are one of the most ubiquitous metallocofactors known in nature. They can attain three oxidation states, $[4Fe\text{-}4S]^{n+}$ with n = 1, 2, or 3, of which the $[4Fe\text{-}4S]^{2+}$ form is the most prevalent redox state. Because the $[4Fe\text{-}4S]^{2+}$ clusters have a diamagnetic (S = 0) ground state, they are not amenable to EPR characterization. We have therefore explored the possibility of using cryoreduction to convert the EPR-silent $[4Fe\text{-}4S]^{2+}$ form to the EPR-active $[4Fe\text{-}4S]^{+}$ form. While this approach has not been successful in the studies of several [4Fe-4S]-containing proteins, we have recently been able to reduce the $[4Fe\text{-}4S]^{2+}$ cluster of TsrM, an enzyme that belongs to the superfamily of the so-called radical-SAM enzymes. These enzymes reductively cleave *S*-adenosyl-methionine (SAM) to yield methionine and a 5'-deoxyadenos-5'-yl radical intermediate using an electron derived from a reduced $[4Fe\text{-}4S]^{+}$ cluster. Studies on TsrM are ongoing and we anticipate that the cryoreduction experiments will contribute to our identification of the cofactors of TsrM and how its substrates interact with the [4Fe-4S] cluster.

Outreach and Training in Bioinorganic Chemistry

Because the research in bioinorganic chemistry is so diverse, the training of students and postdocs is of great significance in this field. The Penn State Bioinorganic group has offered large training workshops in 2012 and 2014, at which participants can learn about many methods employed in the field. The cryoreduction technique was also offered as one of 20 experimental methods during these workshops. Mrs. Candace Davison from the Breazeale Nuclear Reactor and members of the bioinorganic group have jointly trained approximately 60 undergraduate students, graduate students, postdocs, technicians, and faculty in the use of the method.

References

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3. S. F. Ye, J. C. Price, E. W. Barr, M. T. Green, J. M. Bollinger, C. Krebs, and F. Neese, J Am Chem Soc **132**, 4739-4751 (2010).

Publications

1. J. C. Price, E. W. Barr, B. Tirupati, J. M. Bollinger, and C. Krebs, "The first direct characterization of a high-valent iron intermediate in the reaction of an alpha-ketoglutarate-dependent dioxygenase: A high-spin Fe(IV) complex in taurine/alpha-ketoglutarate dioxygenase (TauD) from Escherichia coli," Biochemistry-Us **42**, 7497-7508 (2003).
2. S. F. Ye, J. C. Price, E. W. Barr, M. T. Green, J. M. Bollinger, C. Krebs, and F. Neese, "Cryoreduction of the NO-Adduct of Taurine:alpha-Ketoglutarate Dioxygenase (TauD) Yields an Elusive {FeNO}(8) Species," J Am Chem Soc **132**, 4739-4751 (2010).