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The Manganese Ion of the Heterodinuclear Mn/Fe Cofactor in *Chlamydia trachomatis* Ribonucleotide Reductase R2c Is Located at Metal Position 1

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Supporting Information

ABSTRACT: The essential catalytic radical of Class-I ribonucleotide reductase is generated and delivered by protein R2, carrying a dinuclear metal cofactor. A new R2 subclass, R2c, prototyped by the *Chlamydia trachomatis* protein was recently discovered. This protein carries an oxygen-activating heterodinuclear Mn(II)/Fe(II) metal cofactor and generates a radical-equivalent Mn(IV)/Fe(III) oxidation state of the metal site, as opposed to the tyrosyl radical generated by other R2 subclasses. The metal arrangement of the heterodinuclear cofactor remains unknown. Is the metal positioning specific, and if so, where is which ion located? Here we use X-ray crystallography with anomalous scattering to show that the metal arrangement of this cofactor is specific with the manganese ion occupying metal position 1. This is the position proximal to the tyrosyl radical site in other R2 proteins and consistent with the assumption that the high-valent Mn(IV) species functions as a direct substitute for the tyrosyl radical.

Ribonucleotide reductase (RNR) performs the reduction of all four ribonucleotides to their corresponding deoxyribonucleotide via a radical mechanism. The radical generating R2 protein of Class-I RNR uses a dimetal carboxylate cofactor to produce, store and reversibly deliver the essential radical to the catalytic R1 subunit.[1−3] Class Ic R2 proteins (R2c), prototyped by the *Chlamydia trachomatis* R2c (*Ct*R2c), is a recently discovered group that utilize a heterodinuclear Mn/Fe cofactor. Upon reaction with molecular oxygen, the reduced Mn(II)/Fe(II) site in *Ct*R2c generates a Mn(IV)/Fe(III) radical equivalent oxidation state that is used in place of the standard tyrosyl radical, used by subclasses Ia and Ib.[4−7] Class Ic R2 proteins carry a Phe residue in the position where the radical-harboring Tyr is found in subclasses Ia and Ib (Figure 1).[5,8] R2c sequences are found in several bacteria and archaea, in particular among extremophiles and pathogens.[8] Despite extensive biochemical, spectroscopic and computational studies, the arrangement of the heterodinuclear cofactor remains unknown. It is thus unclear if the positioning of the metals is specific, and if so, at which position they are located.

Here we use X-ray crystallography with anomalous scattering to show that the manganese ion in *Ct*R2c is specifically located in position 1 of the metal site. This position is the one neighboring the otherwise radical-harboring tyrosine in Class-Ia and -Ib R2 proteins and connected to the proposed radical transfer path between proteins R2 and R1.

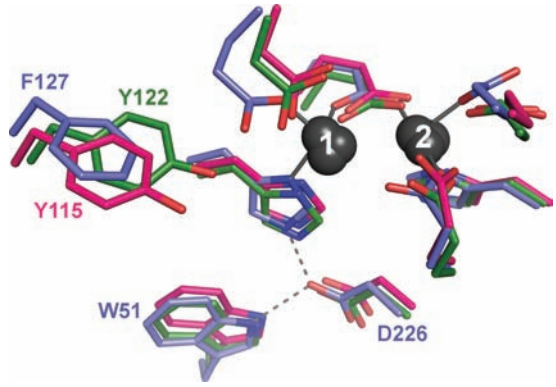

Figure 1. Superposition of R2 protein metal sites: Class Ia (green, *Escherichia coli*, PDB id 1MXR),[15] Class Ib (red, *Corynebacterium ammoniagenes*, PDB id 3MJO),[16] and Class Ic (blue, *Chlamydia trachomatis*, PDB id 1SYY).[5] Metal positions are indicated as well as the radical harboring tyrosine residues of subclasses Ia and Ib (Y122, Y115), in subclass Ic this position is occupied by a Phe (F127). Part of the hydrogen-bonded chain of conserved residues, proposed to be involved in radical transfer between proteins R2 and R1 is also indicated (D226, W51, *C. trachomatis* numbering).

Production of *Ct*R2c by *Escherichia coli* overexpression in standard rich medium yields a very low Mn content with accompanying low activity.[7,9] To obtain high activity the protein either has to be produced in Mn-supplemented media or by preparation of metal depleted protein followed by metal reconstitution while minimizing the formation of a stable homodinuclear diiron site.[7,10] Depending on the method of preparation, a fraction of the sample always contains the diiron site, which forms in parallel with the highly active heterodinuclear Mn/Fe cofactor.[5,7,10−12] For this study the protein was produced by overexpression in *E. coli* grown in Mn-supplemented rich medium, as previously described.[9,12] Metal content of the *Ct*R2c protein and ribonucleotide reductase activity with *C. trachomatis* R1 was confirmed to be at par with previously published data for this method of preparation. The numbers differed slightly between batches with a specific

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activity between 100 and 150 nmol min^{-1} mg^{-1} and a metal content of 1−1.45 equiv of Fe and 0.35−0.45 equiv of Mn per polypeptide. Higher manganese content corresponded to higher activity, as also previously observed.[7,9−12] Crystallization of the protein as described previously[13] yielded crystals diffracting to high resolution (1.5 Å, Supporting Information (SI) Table 1). Anomalous dispersion measurements revealed that an anomalous scatterer, most likely Pb(II) from the crystallization solution, was present in the dinuclear site. The strong Pb anomalous signal precluded a direct and unambiguous observation of a manganese anomalous signal. For a detailed description and analysis of this data see SI.

To be able to directly observe the anomalous signal from both Mn and Fe, and thus the complete heterodinuclear cofactor in the protein, a crystallization condition not including lead was identified (3 μL of protein at 3 mg/mL in 8 mM Tris-HCl buffer at pH 7.5, 1 mM $MnCl_2$, 1 mM $MgCl_2$ was mixed with 0.5 μL 0.1 M MES pH 6.2, 9% PEG 20K and left to equilibrate over the same solution). This condition produced crystals diffracting to lower resolution, 3.2 Å, but still sufficient to measure the Mn and Fe anomalous signals in the absence of any disturbing anomalous scatterers. Anomalous dispersion data were collected at λ = 1.7 Å (high energy side of Fe K-edge) 1.85 Å (Between Mn and Fe K-edges) and 1.92 Å (low energy side of Mn K-edge)(SI Figure 1). At λ = 1.85 Å Mn, but not Fe, displays an anomalous signal. As shown in Figure 2a, Mn is exclusively located in position 1 of the metal site with no signal above noise level in position 2. At λ = 1.7 Å both Mn and Fe, display anomalous signals. As shown in Figure 2b, an anomalous signal, not present at λ = 1.85 Å appears in position 2 of the metal site showing the presence of iron in this position. The data collected at λ = 1.92 Å showed no anomalous signal above noise level in the dinuclear site, confirming that Mn and Fe are the only contributors to the signals observed at λ = 1.85 and 1.7 Å. From this data it is not possible to define if a low occupancy of iron is present in site 1, which would indicate that a fraction of the sample contained a diiron site, as generally observed.[5,7,10−12] The existence of a diiron site at partial occupancy is however directly observed in the data from the Pb-containing crystal, showing that site 2 is virtually fully occupied by Fe and that site 1 contains a smaller, but detectable, amount of iron (SI). The limited resolution of the lead-free crystals prohibits a detailed analysis of the metal coordination or redox-dependent structural changes of the heterodinuclear cofactor. Work to obtain high-resolution structures of the fully occupied site for such studies is ongoing.

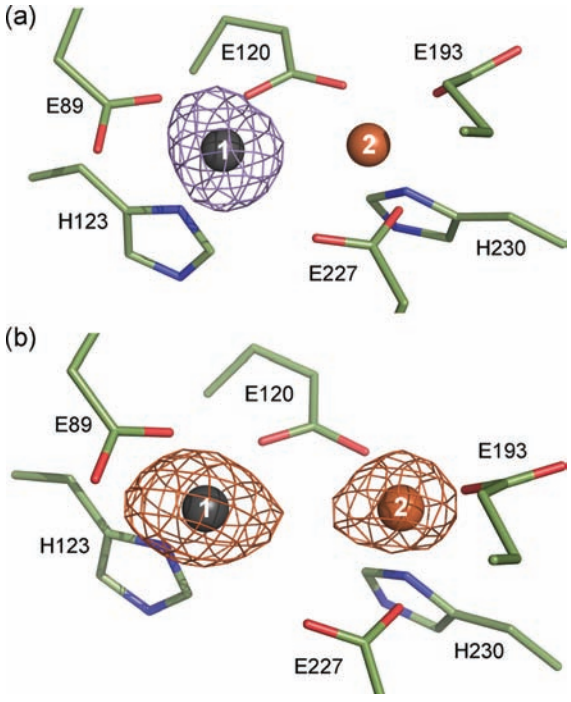

Figure 2. (a) Anomalous difference map at λ = 1.85 Å where Mn, but not Fe, display anomalous scattering, contoured at 0.08 e $Å^{-3}$. (b) Anomalous difference map at λ = 1.7 Å where both Mn and Fe display anomalous scattering, contoured at 0.08 e $Å^{-3}$. The data show that Mn is present in metal position 1 but not metal position 2. The appearance of an anomalous signal in position 2 at λ = 1.7 Å shows that Fe is present in this position. The absence of any anomalous scatterers other than Fe and Mn was verified by a data set collected at λ = 1.92 Å.

Together, these data show that production of the protein, also in Mn-enriched media, results in only Fe occupying metal position 2. Moreover, Fe-binding in position 2 is not displaced either by incubation of the protein with 1 mM Mn(II) or 0.33 mM Pb(II) and thus likely of high affinity. Position 1, on the other hand, can accommodate Mn, Fe as well as Pb. The arrangement with Mn in site 1 and Fe in site 2 of the active cofactor is the same arrangement as observed in the recently discovered R2lox protein from *Mycobacterium tuberculosis*, which also possesses a heterodinuclear Mn/Fe cofactor.[14] From a functional perspective, it is interesting to note that the high-valent Mn(IV) ion is located in the position proximal to the radical harboring tyrosine in subclasses Ia and Ib. The metal ion in this position is directly coordinated by a residue from the hydrogen-bonded chain of conserved residues, proposed to take part in the radical transfer between proteins R2 and R1.[1] Positioning the radical-initiating species in this site could suggest that only minimal additional adaptations are required in the radical transfer mechanism compared to the tyrosyl radical containing RNRs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting results, figures, experimental procedures, and crystallographic statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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