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A Manganese(IV)/Iron(III) Cofactor in Chlamydia trachomatis Ribonucleotide Reductase

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In a conventional class I ribonucleotide reductase (RNR), a diiron(II/II) cofactor in the R2 subunit reacts with oxygen to produce a diiron(III/IV) intermediate, which generates a stable tyrosyl radical (Y•). The Y• reversibly oxidizes a cysteine residue in the R1 subunit to a cysteylnyl radical (C•), which abstracts the 3′-hydrogen of the substrate to initiate its reduction. The RNR from Chlamydia trachomatis lacks the Y•, and it had been proposed that the diiron(III/IV) complex in R2 directly generates the C• in R1. By enzyme activity measurements and spectroscopic methods, we show that this RNR actually uses a previously unknown stable manganese(IV)/iron(III) cofactor for radical initiation.

Ribonucleotide reductases (RNRs) provide all organisms with 2′ deoxyribonucleotides for DNA synthesis (1, 2). All known RNRs are thought to initiate ribonucleotide reduction by using a cysteine thiol radical to abstract the hydrogen atom from the 3′-carbon (3, 4). Three distinct strategies to generate the initiating cysteinylnyl radical (C•) have been described and are, in part, the basis for division of the RNRs into three classes. Class II and III RNRs use strategies involving the 5′-deoxyadenosyl radical, generated either by homolysis of the Co-C bond of 5′-deoxyadenosylcob(III)alamin (class II) or by reductive cleavage of the 5′-C–S bond of S-adenosyl-L-methionine by a separate activase protein (class III), as the ultimate oxidant for cysteine activation. The 5′-deoxyadenosyl radical either generates the C• directly (class II) or generates a stable glycolal radical (G•) that reversibly oxidizes the cysteine (class III) (3, 5, 6).

In a conventional class I RNR (e.g., from Homo sapiens, Saccharomyces cerevisiae, or aerobic Escherichia coli), a binuclear iron center in its cofactor subunit, R2, reacts with oxygen to oxidize a tyrosine residue by one electron to a stable tyrosyl radical (Y•). The Y• in R2 generates the C• in the catalytic subunit, R1, where nucleotide reduction occurs (7).

An unexpected adaptation of the class I functional architecture was revealed by the recent characterization of the RNR from the bacterium Chlamydia trachomatis (8), an obligate intracellular parasite and important human pathogen. The presence of a phenylalanine in place of the tyrosine residue in R2 that normally harbors the essential initiating Y• was revealed first by sequence comparisons (8) and subsequently by x-ray crystallography (9). Consistent with these findings, no evidence for a Y• was found in biochemical studies (9–11). Sequences of R2 genes from other organisms revealed that the absence of the Y• is not specific to the chlamydial RNRs (9). Notably, the presence of genes encoding such R2 proteins in the genomes of other pathogens (e.g., Mycobacterium tuberculosis) suggested that the novel RNRs might have arisen as an adaptation to the host’s immune response (9) and might present specific targets for design of new antibacterial drugs.

To explain how the C. trachomatis RNR can function without the essential Y•, Nordlund, Gräslund, and co-workers suggested that an Fe2†III cofactor might be produced in R2 by reaction of the Fe2†III cluster with O2 and directly oxidize the cysteine in R1 (9). Biochemical studies by Gräslund and co-workers supported this hypothesis by demonstrating both stabilization of the reactive Fe2†IV complex in the presence of the other reaction components (R1, substrate, and allosteric effector) (10) and induction of the Fe2†IV state from Fe2†III-R2 under these turnover conditions (11).

Although the reported induction and stabilization of the Fe2†IV-R2 complex were consistent with the earlier Nordlund/Gräslund hypothesis, the fact that this form was never enriched to greater than ~30% of the total protein (10) left open the possibility that a different form was responsible for the modest activity observed. After preparing His6-affinity-tagged forms of C. trachomatis R2 and the N-terminally truncated Δ1 to 248-R1 (12) reported by McCarty and co-workers to be more stable than full-length R1 (8), we noted a distinct lack of correlation between the catalytic activities and iron contents of different preparations of R2 (13).

By reductive chelation of iron from the purified protein and subsequent dialysis against ethylenedinitrilotetraacetate (EDTA) (12), R2 was isolated with less than 0.02 equivalents (equiv) iron (14) and very low catalytic activity [velocity (v)/[R2] ≤ 0.01 s−1] (15). The metal-depleted R2 so obtained was not detectably activated by addition of 2 equiv of Fe2+. By contrast, addition of both Fe2+ and Mn2+ was found to activate the metal-depleted R2 by a factor of more than 50 (Fig. 1). A Mn2+/Fe2+ ratio of unity gave maximal activation (Fig. 1A), and a total of two divalent metal ions per monomer was sufficient for ~85% of maximal activation (Fig. 1B).

The results in Fig. 1, in particular the 1:1 Mn:Fe ratio giving maximal activity, suggest the use of a Mn/Fe cofactor rather than a Fe2+ cofactor by C. trachomatis RNR. Because the raison d’être of the R2 subunit and its metallocofactor is to transiently oxidize the cysteine residue in R1 by one electron (7), the fully reduced complex formed upon addition of the divalent metal ions to the protein (Mn2+/Fe2+R2) cannot be active but, by analogy with other class I RNRs, might react with O2 to produce an oxidized state that functions in catalysis. Indeed, we observed no turnover after addition of an O2-free solution containing R2, Mn2+, and Fe2+ (0.75 equiv of royalty from the marketing of dicamba-resistant technology if crop seeds containing the resistance gene are marketed by Monsanto, the licensee of this technology from the University of Nebraska.

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Tables S1 to S3

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The active form exhibits no obvious electron paramagnetic resonance (EPR) signal (in X-band, perpendicular mode). A sample prepared with 0.5 equiv Fe and 1.0 equiv Mn (to disfavor formation of Fe\(^{III/III}\)) product exhibits a Mössbauer quadrupole doublet at 4.2 K and zero field (Fig. 2A). The parameters (isomer shift (δ) = 0.52 mm s\(^{-1}\); quadrupole splitting parameter (ΔE\(\text{Q}\)) = 1.32 mm s\(^{-1}\)) establish that the iron site is converted to Fe\(^{III}\) by the reaction with O\(_2\). The sharp [line width (\(\Gamma\)) = 0.30 mm s\(^{-1}\)] doublet in zero magnetic field and marked broadening in a weak (53 mT) field (Fig. 2B) reveal that this complex has a paramagnetic ground state with an integer value of the total electron spin quantum number, \(S_{\text{Total}}\). This characteristic distinguishes the C. trachomatis Mn\(^{IV}/Fe^{III}\)-R2 product from the previously characterized product Fe\(^{III/III}\)-clusters in other R2 proteins, which are diamagnetic (\(S_{\text{Total}} = 0\)) as a result of antiferromagnetic coupling between the two \(S = 5/2\) Fe\(^{III}\) ions. The presence of a Mn ion coupled to the Fe\(^{III}\) site is demonstrated by the EPR spectra of the one-electron reduced form of the complex produced by a brief (~2 min at 22°C) treatment with 20 mM dithionite. These spectra show hyperfine coupling to both an I (nuclear spin quantum number) = 5/2 \(^{55}\)Mn nucleus (Fig. 3A) and an \(I = 1/2\) \(^{57}\)Fe nucleus (compare Fig. 3, A and B). The EPR spectra establish that the reduced form has a coupled Mn/Fe cluster with \(S_{\text{Total}} = 1/2\). The Mössbauer spectrum of the reduced complex acquired at zero field and 190 K (Fig. 2C) is a broad (\(\Gamma \sim 0.5\) mm s\(^{-1}\)) quadrupole doublet (16) with parameters (δ = 0.43 mm s\(^{-1}\), ΔE\(\text{Q}\) = 0.81 mm s\(^{-1}\)) that indicate that the Fe site remains in the \(+III\) oxidation state. Thus, the dithionite treatment reduces the Mn site but not the Fe\(^{III}\) site of the active state. The reduced Mn site must have an even number of valence electrons for coupling with the odd-electron Fe\(^{III}\) site to give \(S_{\text{Total}} = 1/2\). Mn\(^{III}\) (\(S = 2\)) is the only chemically reasonably possible, establishing that the Mn in the active state is \(+IV\). Most likely, antiferromagnetic coupling between the Mn\(^{IV}\) (\(S_{\text{Mn}} = 3/2\)) and high-spin Fe\(^{III}\) (\(S_{\text{Fe}} = 5/2\)) ions gives \(S_{\text{Total}} = 1\), consistent with the observed Mössbauer characteristics (broadening in a 53-mT magnetic field) of the active form.

The EPR spectrum at 4 K of the Mn\(^{IV}/Fe^{III}\)-R2 is perturbed in the presence of R1, cytidine 5'-diphosphate (CDP), and adenosine 5'-triphosphate (ATP) (compare Fig. 3, A and C). This observation indicates that binding of R1 to Mn\(^{IV}/Fe^{III}\)-R2 (and possibly binding of the nucleotides to the R1-R2 complex) affects the structure of the buried cofactor, a phenomenon not previously observed in a class I RNR. The spectra at 14 K (Fig. 3, D to G, solid curves) are particularly sharp and featured, and show hyperfine coupling to \(^{57}\)Fe (compare Fig. 3, D and E) as well as \(^{55}\)Mn. By simulation of the spectrum of the \(^{57}\)Fe-containing sample (Fig. 3D, dashed curve), the g-tensor of the \(S = 1/2\) ground state (2.030, 2.020, 2.015) and the \(^{55}\)Mn hyperfine coupling tensor, \(A_{\text{Mn}}\) ([269, 392, 314] MHz), were determined. The marked anisotropy of \(A_{\text{Mn}}\) is consistent with the assigned \(+III\) oxidation state (17, 18). Additional evidence for the \(+III\) iron valence is provided by the \(^{57}\)Fe hyperfine coupling (Fig. 3E, solid curve), which can be reproduced (Fig. 3E, dashed curve) with an isotropic \(A_{\text{Fe}}\): \([-64.5, -64.5, -64.5]\) MHz of high-spin Fe\(^{III}\) (19).

The substrate analog, 2'-azido 2'-deoxyadenosine 5'-diphosphate (N3-ADP), was used to confirm the conclusion that the Mn\(^{IV}/Fe^{III}\) cluster is the functional cofactor. It has been shown that treatment of a class I or class III RNR with a 2'-azido--substituted nucleotide results in irreversible loss of the C* generator (20, 21) as a result of aberrant reactions, beginning with loss of the azido moiety (either as \(N_3^-\) or \(N_3\)) from the initial 3'-centered radical (22–24). Thus, the \(\gamma\) or \(\gamma\) is irreversibly reduced (20, 21) instead of being regenerated, as it is at the end of a normal turnover. We predicted from these precedents that treatment of C. trachomatis RNR should lead to irreversible conversion of the EPR-silent Mn\(^{IV}/Fe^{III}\) cluster to the EPR-active Mn\(^{IV}/Fe^{II}\) state. Indeed, treatment with N3-ADP generates the same EPR signal seen upon dithionite reduction of Mn\(^{IV}/Fe^{III}\)-R2 in the presence of R1, CDP, and ATP (Fig. 3, F and G). This signal does not develop when the inactivator is replaced by the natural substrate, CDP. The additional features in the 3330 to 3420 G region (marked by arrows; see also Fig. S1) are attributable to the nitrogen-centered radical previously shown to accumulate during N3-NDP-mediated inactivation of conventional class I RNRs (24–26). The formation of the Mn\(^{IV}/Fe^{III}\) cluster and free radical upon reaction with N3-ADP confirms the activity of the Mn\(^{IV}/Fe^{III}\) cluster.

Scheme 1 summarizes our working hypothesis for how the C. trachomatis RNR functions without a \(\gamma\) or \(\gamma\) initiator. By using manganese in place of one of the iron of the conventional R2 metal center, C. trachomatis R2 is able to generate an oxidized cluster that possesses both kinetic stability and sufficient oxidative potency to generate the \(C^*\) in R1 when triggered to do so by the protein(s). The marked perturbation to the EPR signal of the Mn\(^{IV}/Fe^{III}\) cluster caused by binding of R1 (and perhaps nucleotides) provides a tool not present in conventional class I RNR systems for investigating the triggering process.

We attempted to reconcile our conclusion that Mn\(^{IV}/Fe^{III}\)-R2 is the active form with previous observations suggesting that Fe\(^{III/IV}\)-R2 is active (9–11). We were unable to reproduce the reported induction of the Fe\(^{III/IV}\) complex from Fe\(^{III/III}\)-R2 under turnover conditions (11), even though we did verify that turnover was occurring (at 4 to 5% of the rate of the Mn/Fe-activated R2). Addition of a solution containing R2 and 1.5 equiv Fe\(^{III}\) to a solution containing O\(_2\), R1 (2 equiv relative to R2), CDP, and ATP at 22°C did, as previously reported by the Gräslund group (10), result in generation and stabilization.

![Fig. 1. Dependence of the catalytic activity of C. trachomatis R2 on the equivalencies of Mn\(^{IV}\) and Fe\(^{III}\) (A) at a constant total metal equivalency of 2 per protein monomer and (B) at a constant mole fraction of 0.5 for each metal. Each data point is the mean of three trials, and the error bars are the standard deviations.](image-url)

![Fig. 2. Mössbauer spectra of active Mn\(^{IV}/Fe^{III}\)-R2 and the product of its dithionite reduction, Mn\(^{IV}/Fe^{III}\)-R2. (A and B) Spectra of a sample enriched in Mn\(^{IV}/Fe^{III}\)-R2 (12) acquired at 4.2 K in zero magnetic field (A) and with a 53-mT field applied parallel to the \(\gamma\) beam (B). (C) Spectrum at 190 K and zero field of a sample enriched in Mn\(^{IV}/Fe^{III}\)-R2 by dithionite reduction of Mn\(^{IV}/Fe^{III}\)-R2 (12). The solid lines in (A) and (C) are theoretical quadrupole doublets for Mn\(^{IV}/Fe^{III}\)-R2 and Mn\(^{IV}/Fe^{II}\)-R2, respectively, with parameters as in the text.](image-url)
Moreover, replacement of CDP with N3-ADP in the experiment giving the stabilized FeII cluster by dithionite reduction of R2 might result from a Mn competence of the FeII complex did not accelerate decay of this product from these samples that turned over was occurring at the same rate in the experiments. In (D) to (G), a minor contribution from free MnII has been removed by subtraction of the field axis, 2048 points; scans per spectrum, 10. The sharp signal at 3380 G is from the spectrometer wave power, 0.20 mW; modulation amplitude, 4 G; scan time, 334s; time constant, 167 ms; resolution of temperature, 14.0 ± 0.2 K, micro-
temperature indicated on Oxford cryostat); conditions were [(A) to (C)] temperature of 100 kHz. Other spectrometer conditions were [A] to (C) temperature, 4 K (nominal temperature indicated on Oxford cryostat); microwave power, 20 mW; modulation amplitude, 10 G; scan time, 167 s; time constant, 167 ms; resolution of field axis, 1024 points; scans per spectrum, 1; [(D) to (G)] temperature, 14.0 ± 0.2 K, microwave power, 0.20 mW; modulation amplitude, 4 G; scan time, 334 s; time constant, 167 ms; resolution of field axis, 2048 points; scans per spectrum, 10. The sharp signal at 3380 G is from the spectrometer cryostat and is prominent in (A) to (C) because of the low temperature and high power used for these spectra. In (D) to (G), a minor contribution from free MnII has been removed by subtraction of the spectra of control samples either not treated with dithionite [for (D) and (E)] or treated with CDP instead of N3-ADP [for (F) and (G)]. The dashed, lighter-colored traces in (D) to (G) are simulations of the spectra of MnIII/FeII-R2-R1 (12) with the g and A tensors as in the text.

**Fig. 3.** X-band EPR spectra illustrating formation of the MnIII/FeIII cluster by dithionite reduction of active MnIV/FeII-R2 (A to E) or by treatment of the holoenzymes (MnIV/FeII-R2-R1+ATP) with N3-ADP (F and G). The dithionite reduction was carried out either in the absence [(A) and (B)] or in the presence [(C) to (E)] of R1, CDP, and ATP. For (A), (C), (D), and (F), the R2 samples were prepared with natural-abundance iron (91.7% 56Fe with I = 0); for (B), (E), and (G), 57Fe-enriched iron (95%, I = 1/2) was used. All spectra had a microwave frequency of 9.45 GHz and a modulation frequency of 100 kHz. Other spectrometer conditions were the same as those in (A) to (C). Temperature, 4 K (nominal temperature indicated on Oxford cryostat); microwave power, 20 mW; modulation amplitude, 10 G; scan time, 167 s; time constant, 167 ms; resolution of field axis, 1024 points; scans per spectrum, 1; [(D) to (G)] temperature, 14.0 ± 0.2 K, microwave power, 0.20 mW; modulation amplitude, 4 G; scan time, 334 s; time constant, 167 ms; resolution of field axis, 2048 points; scans per spectrum, 10. The sharp signal at 3380 G is from the spectrometer cryostat and is prominent in (A) to (C) because of the low temperature and high power used for these spectra. In (D) to (G), a minor contribution from free MnII has been removed by subtraction of the spectra of control samples either not treated with dithionite [for (D) and (E)] or treated with CDP instead of N3-ADP [for (F) and (G)]. The dashed, lighter-colored traces in (D) to (G) are simulations of the spectra of MnIII/FeII-R2-R1 (12) with the g and A tensors as in the text.
by Escherichia coli in response to oxidative stress of a Mn-dependent paralog to the constitutively expressed, Fe-dependent superoxide dismutase (SOD). Examination of the reactivity of the distinct Mn/Fe cofactor toward these oxidants may thus provide a biochemical rationale for its evolution.

**References and Notes**

12. Materials and methods are available as supporting material on Science Online.
13. For example, preparations from E. coli cultures grown in rich medium had the same iron content as preparations from iron-supplemented minimal medium (~0.75 equiv; all metal equivalences and activities are on a per monomer basis) but ~10 times the activity (velocity (ν/ν[R2]) = 0.035 ± 0.01 s⁻¹, compared with 0.003 ± 0.001 s⁻¹). Conversely, R2 from rich medium to which the cell-permissive Fe²⁺ chelator, 1,10-phenanthroline, was added immediately before induction of overexpression emerged with much less iron (<0.05 equiv) but ~70% of the activity (ν/ν[R2]) = 0.025 ± 0.01 s⁻¹) of the R2 from equivalent cultures lacking the chelator.
14. All metal equivalences and activities for the homodimeric R2 protein are reported on a per monomer basis.
15. To reduce the residual R2 activity to this low level, it was necessary also to dialyze the R1 used in the activity assay against EDTA.
16. The S = 1/2 complex exhibits a magnetically split Mössbauer spectrum at low temperature, but the use of this higher temperature makes electronic relaxation fast compared with the [35Fe] nuclear precession frequency and collapses the spectrum into a quadrupole doublet.
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**Supporting Online Material**

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Materials and Methods

Figs. S1 to S3

References

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**Probing Transcription Factor Dynamics at the Single-Molecule Level in a Living Cell**

Johan Elf, Gene-Wei Li, X. Sunney Xie

Transcription factors regulate gene expression through their binding to DNA. In a living Escherichia coli cell, we directly observed specific binding of a lac repressor, labeled with a fluorescent protein, to a chromosomal lac operator. Using single-molecule detection techniques, we measured the kinetics of binding and dissociation of the repressor in response to metabolic signals. Furthermore, we characterized the nonspecific binding to DNA, one-dimensional (1D) diffusion along DNA segments, and 3D translocation among segments through cytoplasm at the single-molecule level. In searching for the operator, a lac repressor spends ~90% of time nonspecifically bound to and diffusing along DNA with a residence time of ~5 milliseconds. The methods and findings can be generalized to other nucleic acid binding proteins.

In all kingdoms of life, transcription factors (TFs) regulate gene expression by site-specific binding to chromosomal DNA, preventing or promoting the transcription by RNA polymerase. The lac operon of Escherichia coli, a model system for understanding TF-mediated transcriptional control (1), has been the subject of extensive biochemical (2–4), structural (5), and theoretical (6, 7) studies since the seminal work by Jacob and Monod (8). However, the in vivo kinetics of the lac repressor, and all other TFs, has only been studied indirectly by monitoring the regulated gene products. Traditionally, this was done on a population of cells (9), in which unsynchronized gene activity among cells masks the underlying dynamics. Recent experiments on single cells allow investigation of stochastic gene expression (10–15). However, direct observation of TF-mediated gene regulation (16) remains difficult, because it often involves only a few copies of TFs and their chromosomal binding sites. Here we report on a kinetics study of how fast a lac repressor binds its chromosomal operators and dissociates in response to a metabolic signal in a living E. coli cell.

Single-molecule detection also makes it possible to investigate how a TF molecule searches for specific binding sites on DNA, a central question in molecular biology. Target location by TFs (and most nucleic acid binding proteins) is believed to be achieved by facilitated diffusion, in which a TF searches for specific binding sites through a combination of one-dimensional (1D) diffusion along a short DNA segment and 3D translocation among DNA segments through cytoplasm (17). However, real-time observation in living cells has not been available because of technical difficulties. Here we report on such an investigation, providing quantitative information of the search process.

The lac repressor (Lacl) is a dimer of dimers. Under repressed conditions one dimer binds the major lac operator, O1, and the other dimer binds one of the weaker auxiliary operators, O2 or O3 (18) (Fig. 1A). Lacl binding to O1 prevents RNA polymerase from transcribing the lac operon (lac2ZYA). Upon binding of allolactose, an intermediate metabolite in the lactose pathway, or a nondegradable analog, such as IPTG (isopropyl β-D-thiogalactopyranoside), the repressor’s affinity for the operator is substantially reduced to a level comparable to that of nonspecific DNA interaction (19).

To image the lac repressor, we expressed it from the native chromosomal lacI locus as a C-terminal fusion with the rapidly maturing (~7 min) yellow fluorescent protein (YFP) Venus (A206K) (15, 20) (Fig. 1A). The short maturation time prevents the lac operator sites from being occupied by immature fusion proteins. The C-terminal fusion avoids interference with the N-