Control of metallation and active cofactor assembly in the class Ia and Ib ribonucleotide reductases: diiron or dimanganese?

Control of metallation and active cofactor assembly in the class Ia and Ib ribonucleotide reductases: diiron or dimanganese?

JoAnne Stubbe[1,2] and **Joseph A. Cotruvo Jr.**[1]

[1]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

[2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

Abstract

Ribonucleotide reductases (RNRs) convert nucleotides to deoxynucleotides in all organisms. Activity of the class Ia and Ib RNRs requires a stable tyrosyl radical (Y•), which can be generated by reaction of O_2 with a diferrous cluster on the β subunit to form active diferric-Y• cofactor. Recent experiments have demonstrated, however, that in vivo the class Ib RNR contains an active dimanganese(III)-Y• cofactor. The similar metal binding sites of the class Ia and Ib RNRs, their ability to bind both Mn^{II} and Fe^{II}, and the activity of the class Ib RNR with both diferric-Y• and dimanganese(III)-Y• cofactors raises the intriguing question of how the cell prevents mismetallation of these essential enzymes. The presence of the class Ib RNR in numerous pathogenic bacteria also highlights the importance of manganese for these organisms' growth and virulence.

The seminal experiments on iron sulfur (FeS) cluster biosynthesis and regulation [1,2] have highlighted the requirement for biosynthetic pathways for assembly of metallocofactors, despite the fact that these cofactors are able to self-assemble with varying degrees of success in vitro. FeS clusters play a key role in iron homeostasis in all organisms and are also involved in heme biosynthesis [2]. However, despite extensive studies of these pathways, the mechanisms of iron insertion into any protein remain largely unknown, especially those containing mono- and dinuclear non-heme iron cofactors, such as Fe-superoxide dismutase (SOD), α-ketoglutarate-dependent dioxygenases, methane monooxygenase, and class Ia ribonucleotide reductases (RNRs). This review focuses on efforts to understand the biosynthesis of the tyrosyl radical (Y•) essential for activity of the class Ia and Ib RNRs. Early studies established that both class Ia and Ib RNRs are active in nucleotide reduction with diferric-Y• cofactors [3,4]. However, more recent results suggest that the class Ib RNR, despite its ability to self-assemble an active diferric-Y• cofactor in vitro, possesses an active dimanganese(III)-Y• cofactor in vivo [5–8]. This surprising observation raises the fundamental question of how correct metallation is ensured by biosynthetic pathways inside the cell for these proteins and metalloproteins in general.

Corresponding author: Stubbe, JoAnne. Tel: 617-253-1814. Fax: 617-324-0505. stubbe@mit.edu.

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Diferric-Y• assembly in class Ia RNRs

Ribonucleotide reductases catalyze the conversion of all four nucleotides (NDPs) to the corresponding deoxynucleotides (dNDPs) in all organisms, supplying the monomeric precursors required for DNA replication and repair [9]. The class I RNRs are composed of two types of subunits, α and β. α houses the active site where NDPs are reduced to dNDPs and the binding sites for the (deoxy)nucleotide allosteric effectors that control substrate specificity and the rate of turnover [10]. β possesses dinuclear metallocofactors [11] (Figure 1) which are essential for initiating nucleotide reduction in α by an unprecedented oxidation involving a long-range proton-coupled electron transfer pathway composed of conserved redox active amino acid residues [12]. The class Ia RNR contains a stable diferric-Y• cofactor (half-life of 20 min and 4 days for the human and *E. coli* enzymes, respectively), remarkable in that the half-life of a Y• in solution is <1 ms. The Y• is essential for catalysis and Nature has evolved pathways to biosynthesize this cofactor and to regenerate it (maintenance) when the Y• is reduced (Figure 2a).

The diferric-Y• cofactor can self-assemble in vitro [13] from apo-β, Fe^{II}, O_2, and reductant by an extensively studied mechanism (Figure 2a) [14–17]. The same basic mechanism is expected to operate in vivo, but the requirements for Fe^{II} loading and the donor of the essential reducing equivalent led our laboratory to investigate how these steps occur inside the cell.

The observation that functionally related genes are often organized within operons has provided the first insight into the machinery involved in the biosynthesis and maintenance of the class Ia and Ib RNR cofactors. Bioinformatic analyses of genomes encoding class Ia RNRs revealed that 29% of the operons containing the genes for the two class Ia RNR subunits (*nrdA* and *nrdB*, *E. coli* nomenclature) also contained a gene that codes for a [2Fe2S]-ferredoxin, *yfaE*. While this gene is not essential, an *E. coli* Δ*yfaE* strain grown in minimal media in the presence of hydroxyurea, a scavenger of the Y•, exhibited a growth rate one-third that of the isogenic wild-type strain. In vitro experiments demonstrated that YfaE is able to rapidly reduce the Y•-reduced (met) diferric cluster to a diferrous cluster, which rapidly self-assembles into active cluster upon addition of O_2 [18] (Figure 2a). Further in vitro studies have also suggested that YfaE is competent to deliver the extra reducing equivalent required for cluster assembly [18]. Whole cell EPR and western blotting analyses of *E. coli* in which β manipulated from 4 μM to 3.3 mM have demonstrated that Y• levels can be modulated in vivo, supporting the physiological relevance of the proposed maintenance pathway [19].

Regarding the mechanism of Fe^{II} loading of β, recent evidence in *Saccharomyces cerevisiae* suggests that FeS clusters are involved directly or indirectly in this process as well. The monothiol glutaredoxins Grx3 and Grx4 contain labile, glutathione-ligated [2Fe2S] clusters [20]. Deletion of Grx3/4 leads to cells that contain high cytosolic iron levels, yet Fe-requiring enzymes located in the cytosol and mitochondria that utilize heme, FeS clusters, and diiron clusters are compromised, suggesting that the iron is not readily bioavailable [21]. In the case of the class Ia RNR, for example, both Fe loading and activity are impaired. These results implicate Grx3/4 in cellular Fe metabolism in *S. cerevisiae*, but where these proteins fit into the cluster assembly pathways is still unclear. Whether a protein with a labile FeS cluster can function to deliver only iron to Fe-requiring proteins, an intriguing possibility, remains to be established.

Discovery of a dimanganese(III)-Y• cofactor in the class Ib RNRs

Class Ia and Ib RNRs are structurally homologous but contain different metallocofactors

In contrast to its class Ia RNR, the class Ib RNR of *E. coli* is not expressed in normal aerobic growth but is induced under iron limitation and oxidative stress [22,23]. Its precise role and importance in these conditions are poorly understood, but elevated expression is known to involve the transcription factors NrdR [24] and Fur [25,26], which sense nucleotide and Fe^{II} levels, respectively. Class Ib enzymes are also the primary or only aerobic RNRs for many other prokaryotes, including pathogens such as *Mycobacterium tuberculosis, Staphylococcus aureus*, and *Bacillus anthracis* [27]. The α and β subunits, NrdE and NrdF respectively, are structurally homologous to the class Ia α and β subunits, and the metal-binding residues in β are identical. Class Ib RNRs are distinct, however, in that α lacks the N-terminal "ATP cone" domain involved in controlling overall enzyme activity [4,28]. Another key difference is that organisms containing class Ib RNRs also contain genes, often in the same operon, encoding a flavodoxin-like protein, NrdI, and NrdH, a thioredoxin-like protein characterized as an electron donor to α [29]. The class Ib RNRs of many organisms have been recombinantly expressed in *E. coli* and purified in an apo form [30] or with diferric-Y• cofactor [4]. Diferric-Y• cofactor can also self-assemble in NrdF and support ribonucleotide reduction [5,31], as in the class Ia RNRs.

The self-assembly of active diferric-Y• cofactors in class Ib RNRs and the presence of identical metal ligands in the class Ia and Ib RNRs suggested to many researchers that the class Ib RNR contained a diferric-Y• inside the cell [31]. However, studies of *Corynebacterium ammoniagenes* [32] and *Bacillus subtilis* [33] have demonstrated the essentiality of Mn for growth. Early biochemical studies of *C. ammoniagenes* NrdF suggested the presence of Mn in the active cofactor [34,35], but Y• was not detected and RNR activity was very low. Furthermore, attempts to generate active NrdF in vitro using Mn^{II} and a variety of oxidants, including O_2 and H_2O_2, were unsuccessful [31]. Our recent experiments elucidating the role of NrdI in the *E. coli* class Ib system and studies of Auling and coworkers purifying *C. ammoniagenes* NrdF from its native organism have both established the activity of a dimanganese(III)-Y• (Mn^{III}_2-Y•) cofactor.

Dimanganese(III)-Y• cofactor formation in vitro

The annotation of NrdI as a flavodoxin found in the *E. coli nrdHIEF* operon suggested that it might play a role in the biosynthetic and maintenance pathways of the class Ib RNR cofactor [30], similar to the proposed role for YfaE in the class Ia RNR (Figure 2a). However, the redox properties of NrdI suggested that it acts as a two-electron reductant [30], unlike typical flavodoxins, which are one-electron reductants. This observation, studies suggesting that NrdI is essential in *S. pyogenes* [36] and *B. subtilis* [37], and the importance of Mn for growth of a number of bacteria [32,33,38], led us to propose that NrdI might provide the oxidant for assembly of a dimanganese-Y• cofactor in NrdF.

Several in vitro observations supported this proposal. First, affinity chromatography of Mn^{II}_2NrdF and tagged NrdI indicated a relatively tight interaction [5]. Second, when Mn^{II}_2-NrdF was incubated with the fully reduced form of NrdI ($NrdI_{hq}$) in an anaerobic chamber and then exposed to O_2, a dimanganese(III)-Y• cofactor was generated which was active in nucleotide reduction [5]. These and other biochemical studies suggested that the role of $NrdI_{hq}$ in cofactor assembly is to reduce O_2 to either H_2O_2 or HO_2^- [HOO(H)], which is channeled directly to the metal site through a NrdI•NrdF complex.

Support for this oxidant channeling model is provided by recent structures of Mn^{II}_2-NrdF alone and in complex with oxidized NrdI and $NrdI_{hq}$, as well as of Fe^{II}_2-NrdF [39]. These structures have also provided insight about the role of the carboxylate ligands of the metal

clusters in providing O_2 access to the Fe^{II}_2 cluster and HOO(H) access to the Mn^{II}_2 cluster. The Mn^{II}_2 cluster of NrdF has a distinct coordination mode of Glu158, never before observed in any RNR βs or related diiron-carboxylate proteins (compare diferrous and dimanganese(II) forms, Figure 1b), and a unique water coordinated to Mn2. The different positions of Glu158 in Fe^{II}_2- and Mn^{II}_2-NrdF result in different routes for the O_2 and HOO(H) oxidants to access the respective metal sites. In Fe^{II}_2-NrdF, the channel terminates at a hydrophobic pocket trans to His195, while in Mn^{II}_2-NrdF, it ends at the water bound to Mn2 [39] (Figure 3). HOO(H) is therefore proposed to replace the water at Mn2 in the first step of cluster assembly [39]. Thus, the structures offer clues as to the distinct mechanisms of Fe^{III}_2-Y• and Mn^{III}_2-Y• cofactor generation and the role of NrdF in catalyzing both processes with different oxidants.

Dimanganese(III)-Y• cofactor formation in vivo

Nearly three decades of effort have recently culminated in the successful isolation of highly active NrdF from *C. ammoniagenes* by the Auling laboratory [6]. This isolation required the overexpression of NrdF in *C. ammoniagenes* to 5% of total cellular protein and growth in the presence of 185 μM Mn^{II}. The purified protein contained 1.5 Mn/β2 and 0.36 Y•/β2 [6]. Extensive characterization of this protein by EPR spectroscopy revealed a Mn^{III}_2-Y• cofactor with a spectrum very similar to the reconstituted Mn^{III}_2-Y• cofactor in *E. coli* NrdF. A high-resolution crystal structure of the *C. ammoniagenes* NrdF, proposed to be in the Mn^{III}_2 state, has also been reported [6] and shows that Glu158 has undergone a dramatic carboxylate shift in the course of cofactor assembly relative to the *E. coli* Mn^{II}_2 structure (Figure 1b). These structures support the importance of ligand reorganization, first suggested by the Lippard group, in reactions of dinuclear redox centers [40].

Very recent studies in *E. coli* and *B. subtilis* complement the studies in *C. ammoniagenes*. *E. coli* NrdF has been purified from *E. coli* GR536, a strain deficient in all known iron uptake systems and requiring Mn for normal growth [41], using a NrdI affinity column and additional columns. The protein was purified 3000-fold to homogeneity [7] and contained 0.9 Mn and 0.2 Y•/β2, comparable to the levels observed in the in vitro reconstitution experiments and those in *C. ammoniagenes* NrdF. *B. subtilis* NrdF has also recently been purified after upregulation of the entire class Ib RNR operon and shown to contain a Mn^{III}_2-Y• cofactor [8]. In all three cases, Mn loading and Y•/β2 is substoichiometric, perhaps related to the genetic manipulation and growth conditions used to observe the cofactor. These studies together suggest that many class Ib RNRs will use Mn^{III}_2-Y• cofactors. However, the possibility that some organisms use Fe^{III}_2-Y• cofactors cannot be excluded at present and must be evaluated by purification of these proteins from the native organisms, a challenge given the low concentrations of RNR and potential Y• instability.

Mechanism of Mn^{III}_2-Y• cofactor assembly

General mechanisms by which the Mn^{II}_2 cluster is oxidized to the Mn^{III}_2-Y• have been proposed [5,6] based on structural data, model systems [42], and the mechanism of Mn catalases [43]. A salient feature of the working models (Figure 2b) is the oxidation of tyrosine to Y• by a $Mn^{IV}Mn^{III}$ intermediate, similar to intermediate **X** ($Fe^{IV}Fe^{III}$) in the class Ia RNRs (Figure 2a). The requirement of two HOO(H) for cluster assembly and the substoichiometric Y• and Mn^{III} loading of NrdF suggests that a reductase is needed to recycle NrdI's flavin cofactor. This is underscored by studies with antibodies to NrdF and NrdI in *E. coli* and *B. subtilis* [7,8], which show that NrdI is present at substoichiometric levels relative to NrdF and likely functions catalytically inside the cell. The ability to obtain more efficient cluster assembly in vitro is essential for mechanistic studies of Mn^{III}_2-Y• cofactor formation.

Conclusion: Control of metallation in vivo

The metal binding sites of class Ia and Ib βs are identical ans both are active with diferric-Y• cofactors, but class Ib RNRs use a Mn^{III}_2-Y• cofactor in vivo. Mn^{II} binds more tightly than Fe^{II} to *E. coli* NrdB [44], but the Mn^{II}_2 form cannot be converted to an active form containing Y•. These observations emphasize the importance of understanding how the cell ensures correct metallation of metalloenzymes such as the class I RNRs[1].

Culotta and coworkers have suggested, based on their studies of the effects of Mn and Fe homeostasis on (mis)metallation of *S. cerevisiae* MnSOD, that correct metallation of proteins in vivo is governed by tuning of the protein environment for the correct metal in relation to the bioavailabilities of the correct and incorrect metals [47]. These bioavailabilities are largely unknown and organism-dependent, making isolation of proteins from their native organism in the appropriate growth conditions crucial.

Understanding the bioavailable pools of various metals inside the cell requires identifying the transcription factors controlling metal homeostasis and divalent metal transporters permitting metal uptake and their specificities. An additional layer of complexity exists for pathogens, for which the host cell can control metal homeostasis [38]. Key features of the host macrophage's response to an engulfed pathogen are assault by reactive oxygen species like superoxide and nitric oxide and depletion of metals required for the pathogen's survival, especially Fe and Mn [38]. Mn^{II} has been demonstrated to be essential for growth and virulence of a number of pathogens such as *Salmonella enterica* serovar Typhimurium and *S. aureus*, which contain class Ib RNRs [38]. The reasons for this dependence on Mn are unclear, but utilization of Mn^{II} for superoxide dismutation, by phosphate-bound Mn^{II} or by MnSOD, is a commonly cited hypothesis [48,49]. The discovery that most, if not all, class Ib RNRs require Mn, suggests that the class Ib RNRs might provide an additional explanation for this requirement for many pathogens. Experimental tests of this hypothesis and further elucidation of the mechanisms of Fe and Mn homeostasis and trafficking within the cell will shed light on why class Ia RNRs are diiron proteins and class Ib RNRs are dimanganese proteins in vivo.

Research Highlights

- Definitive evidence for a Mn_2 cluster in class Ib RNRs has been recently provided

- NrdI is essential in supplying the oxidant for class Ib dimanganese cluster assembly

- Class Ib RNRs are active with Fe or Mn; control of metal loading is critical in vivo

- Class Ib RNR structures reveal carboxylate shifts during metal center oxidation

References

**Of outstanding interest

[1]In addition, the class Ic RNR of *Chlamydia trachomatis* self-assembles both $Mn^{IV}Fe^{III}$ and $Fe^{IV}Fe^{III}$ cofactors in a metal site structurally similar to those of the Ia and Ib RNRs; however, only the $Mn^{IV}Fe^{III}$ cofactor is active in nucleotide reduction, based on the currently available evidence [45]. The oxidizing equivalent is thus stored in the metal cluster instead of a Y•. In fact, in this and predicted class Ic RNRs, the Tyr oxidized to a Y• in class Ia and Ib RNRs is replaced with a Phe or other hydrophobic residue [46]. Consequently, we think it unlikely that a $Mn^{III}Fe^{III}$-Y• RNR will be discovered.

*Of special interest

1. Johnson DC, Dean DR, Smith AD, Johnston MK. Structure, function, and formation of biological iron-sulfur clusters. Annu. Rev. Biochem. 2005; 74:247–281. [PubMed: 15952888]

2. Lill R, Mühlenhoff U. Maturation of iron-sulfur proteins in eukaryotes: Mechanisms, connected processes, and diseases. Annu Rev Biochem. 2008; 77:669–700. [PubMed: 18366324]

3. Sjöberg BM, Reichard P, Gräslund A, Ehrenberg A. Nature of free-radical in ribonucleotide reductase from *Escherichia coli*. J. Biol. Chem. 1977; 252:536–541. [PubMed: 188819]

4. Jordan A, Pontis E, Atta M, Krook M, Gibert I, Barbé J, Reichard P. A second class I ribonucleotide reductase in *Enterobacteriaceae*: Characterization of the *Salmonella typhimurium* enzyme. Proc. Natl. Acad. Sci. U.S.A. 1994; 91:12892–12896. [PubMed: 7809142]

5. Cotruvo JA Jr. Stubbe J. An active dimanganese(III)-tyrosyl radical cofactor in *Escherichia coli* class Ib ribonucleotide reductase. Biochemistry. 2010; 49:1297–1309. [PubMed: 20070127] ** First demonstration of the formation of a Mn^{III}_2-Y• cofactor in biology, by in vitro reconstitution of the class Ib RNR with Mn^{II}, reduced NrdI, and O_2. Biochemical data suggests formation of a tight NrdI•NrdF complex allowing for oxidant channeling. Based on these results, a working model for Mn^{III}_2-Y• generation through Mn^{III}_2 and $Mn^{IV}Mn^{III}$ intermediates is proposed.

6. Cox N, Ogata H, Stolle P, Reijerse E, Auling G, Lubitz W. A tyrosyl-dimanganese coupled spin system is the native metalloradical cofactor of the R2F subunit of the ribonucleotide reductase of *Corynebacterium ammoniagenes*. J. Am. Chem. Soc. 2010; 132:11197–11213. [PubMed: 20698687] ** First definitive demonstration, by EPR spectroscopy and x-ray crystallography, that a dimanganese(III)-Y• cofactor can be formed in vivo in *C. ammoniagenes* NrdF, complementing the previous in vitro results with *E. coli* NrdF [5]. These results and those of refs. 5,7, and 8 together provide compelling evidence for the physiological relevance of the Mn^{III}_2-Y• cofactor in class Ib RNRs.

7. Cotruvo JA Jr. Stubbe J. *Escherichia coli* class Ib ribonucleotide reductase contains a dimanganese(III)-tyrosyl radical cofactor in vivo. doi: 10.1021/bi101881d.

8. Zhang Y, Stubbe J. Class Ib ribonucleotide reductase in *Bacillus subtilis* is a dimanganese(III)-tyrosyl radical enzyme. in preparation.

9. Nordlund P, Reichard P. Ribonucleotide reductases. Annu. Rev. Biochem. 2006; 75:681–706. [PubMed: 16756507]

10. Uhlin U, Eklund H. Structure of ribonucleotide reductase protein R1. Nature. 1994; 370:533–539. [PubMed: 8052308]

11. Cotruvo JA Jr. Stubbe J. Class I ribonucleotide reductases: Metallocofactor assembly and repair in vitro and in vivo. Annu Rev Biochem. 2011 doi: 10.1146/annurev-biochem-061408-095817.

12. Stubbe J, Nocera DG, Yee CS, Chang MCY. Radical initiation in the class I ribonucleotide reductase: Long-range proton-coupled electron transfer? Chem. Rev. 2003; 103:2167–2201. [PubMed: 12797828]

13. Atkin CL, Thelander L, Reichard P, Lang G. Iron and free-radical in ribonucleotide reductase - exchange of iron and Mössbauer-spectroscopy of protein-B2 subunit of *Escherichia coli* enzyme. J Biol Chem. 1973; 248:7464–7472. [PubMed: 4355582]

14. Bollinger JM Jr. Edmondson DE, Huynh BH, Filley J, Norton JR, Stubbe J. Mechanism of assembly of the tyrosyl radical dinuclear iron cluster cofactor of ribonucleotide reductase. Science. 1991; 253:292–298. [PubMed: 1650033]

15. Stubbe J, Riggs-Gelasco P. Harnessing free radicals: Formation and function of the tyrosyl radical in ribonucleotide reductase. Trends Biochem. Sci. 1998; 23:438–443. [PubMed: 9852763]

16. Yun D, Garcia-Serres R, Chicalese BM, An YH, Huynh BH, Bollinger JM Jr. (μ-1,2-peroxo)diiron(III/III) complex as a precursor to the diiron(III/IV) intermediate X in the assembly of the iron-radical cofactor of ribonucleotide reductase from mouse. Biochemistry. 2007; 46:1925–1932. [PubMed: 17256972]

17. Shanmugam M, Doan PE, Lees NS, Stubbe J, Hoffman BM. Identification of protonated oxygenic ligands of ribonucleotide reductase intermediate X. J. Am. Chem. Soc. 2009; 131:3370–3376. [PubMed: 19220056]

18. Wu C-H, Jiang W, Krebs C, Stubbe J. YfaE, a ferredoxin involved in diferric-tyrosyl radical maintenance in *Escherichia coli* ribonucleotide reductase. Biochemistry. 2007; 46:11577–11588. [PubMed: 17880186]

19. Hristova D, Wu C-H, Stubbe J. Importance of the maintenance pathway in the regulation of the activity of *Escherichia coli* ribonucleotide reductase. Biochemistry. 2008; 47:3989–3999. [PubMed: 18314964]

20. Li H, Mapolelo DT, Dingra NN, Naik SG, Lees NS, Hoffman BM, Riggs-Gelasco PJ, Huynh BH, Johnson MK, Outten CE. The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. Biochemistry. 2009; 48:9569–9581. [PubMed: 19715344]

21. Mühlenhoff U, Netz DJA, Molik S, Richter N, Uzarska M, Berthet JG, Seubert A, Zhang Y, Stubbe J, Pierrel F, et al. Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster. Cell Metab. 2010; 12:373–385. [PubMed: 20889129]

22. Monje-Casas F, Jurado J, Prieto-Alamo MJ, Holmgren A, Pueyo C. Expression analysis of the *nrdHIEF* operon from *Escherichia coli*. Conditions that trigger the transcript level *in vivo*. J. Biol. Chem. 2001; 276:18031–18037. [PubMed: 11278973]

23. McHugh JP, Rodriguez-Quiñones F, Abdul-Tehrani H, Svistunenko DA, Poole RK, Cooper CE, Andrews SC. Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. J. Biol. Chem. 2003; 278:29478–29486. [PubMed: 12746439]

24. Torrents E, Grinberg I, Gorovitz-Harris B, Lundström H, Borovok I, Aharonowitz Y, Sjöberg BM, Cohen G. NrdR controls differential expression of the *Escherichia coli* ribonucleotide reductase genes. J. Bacteriol. 2007; 189:5012–5021. [PubMed: 17496099]

25. Vassinova N, Kozyrev D. A method for direct cloning of Fur-regulated genes: identification of seven new Fur-regulated loci in *Escherichia coli*. Microbiology. 2000; 146:3171–3182. [PubMed: 11101675]

26. Panosa A, Roca I, Gibert I. Ribonucleotide reductases of *Salmonella* Typhimurium: Transcriptional regulation and differential role in pathogenesis. PLoS ONE. 2010; 5:e11328. [PubMed: 20593029] * A first consideration of the specific physiological roles that the class Ia and Ib RNRs play in *Salmonella* in pathogenesis, including experiments with *nrdAB* and *nrdEF* mutants conducted in macrophage cell lines.

27. Lundin D, Torrents E, Poole AM, Sjöberg BM. RNRdb, a curated database of the universal enzyme family ribonucleotide reductase, reveals a high level of misannotation in sequences deposited to Genbank. BMC Genomics. 2009; 10:589–596. [PubMed: 19995434]

28. Uppsten M, Färnegårdh M, Jordan A, Eliasson R, Eklund H, Uhlin U. Structure of the large subunit of class Ib ribonucleotide reductase from *Salmonella typhimurium* and its complexes with allosteric effectors. J. Mol. Biol. 2003; 330:87–97. [PubMed: 12818204]

29. Jordan A, Aslund F, Pontis E, Reichard P, Holmgren A. Characterization of *Escherichia coli* NrdH. A glutaredoxin-like protein with a thioredoxin-like activity profile. J. Biol. Chem. 1997; 272:18044–18050. [PubMed: 9218434]

30. Cotruvo JA Jr. Stubbe J. NrdI, a flavodoxin involved in maintenance of the diferrictyrosyl radical cofactor in *Escherichia coli* class Ib ribonucleotide reductase. Proc. Natl. Acad. Sci. U.S.A. 2008; 105:14383–14388. [PubMed: 18799738] * The first characterization of a NrdI protein, demonstrating its unusual redox potentials and its specific interaction with NrdF. The results suggested that NrdI could play a role in diferric-Y• maintenance in class Ib RNRs, also proposed in ref. 35. Given that most class Ib RNRs likely contain a Mn^{III}_2-Y• cofactor in vivo, NrdI might also be involved in an analogous pathway for the Mn cofactor.

31. Huque Y, Fieschi F, Torrents E, Gibert I, Eliasson R, Reichard P, Sahlin M, Sjöberg BM. The active form of the R2F protein of class Ib ribonucleotide reductase from *Corynebacterium ammoniagenes* is a diferric protein. J. Biol. Chem. 2000; 275:25365–25371. [PubMed: 10801858]

32. Oka T, Udagawa K, Kinoshita S. Unbalanced growth death due to depletion of Mn^{2+} in *Brevibacterium ammoniagenes*. J. Bacteriol. 1968; 96:1760–1767. [PubMed: 5726310]

33. Que Q, Helmann JD. Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol. Microbiol. 2000; 35:1454–1468. [PubMed: 10760146]

34. Willing A, Follmann H, Auling G. Ribonucleotide reductase of *Brevibacterium ammoniagenes* is a manganese enzyme. Eur. J. Biochem. 1988; 170:603–611. [PubMed: 2828045]

35. Fieschi F, Torrents E, Toulokhonova L, Jordan A, Hellman U, Barbé J, Gibert I, Karlsson M, Sjöberg BM. The manganese-containing ribonucleotide reductase of *Corynebacterium ammoniagenes* is a class Ib enzyme. J. Biol. Chem. 1998; 273:4329–4337. [PubMed: 9468481]

36. Roca I, Torrents E, Sahlin M, Gibert I, Sjöberg BM. NrdI essentiality for class Ib ribonucleotide reduction in *Streptococcus pyogenes*. J. Bacteriol. 2008; 190:4849–4858. [PubMed: 18502861]

37. Kobayashi K, Ehrlich SD, Albertini A, Amati G, Andersen KK, Arnaud M, Asai K, Ashikaga S, Aymerich S, Bessieres P, et al. Essential Bacillus subtilis genes. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:4678–4683. [PubMed: 12682299]

38. Papp-Wallace KM, Maguire ME. Manganese transport and the role of manganese in virulence. Annu. Rev. Microbiol. 2006; 60:187–209. [PubMed: 16704341] * An excellent review of the key role of Mn in pathogenic organisms, raising important issues in the design and interpretation of experiments aimed at elucidating specificity of metal transporters and the roles of metals in vivo, in particular Mn and Fe. The work highlights the critical role that the Nramp1 status of the host cell line or animal model on the metal-dependent phenotypes observed.

39. Boal AK, Cotruvo JA Jr. Stubbe J, Rosenzweig AC. Structural basis for activation of class Ib ribonucleotide reductase. Science. 2010; 329:1526–1530. [PubMed: 20688982] ** Structures of the NrdI•NrdF complex strongly support the general model outlined in ref. 5 for involvement of NrdI in assembly of the Mn^{III}_2-Y• cofactor, in particular for oxidant channeling. This work also reveals an unprecedented coordination geometry for the dimanganese(II) cluster, which, along with ref. 6, highlights the importance of carboxylate shifts in reactivity of dinuclear metal clusters.

40. Rardin RL, Tolman WB, Lippard SJ. Monodentate carboxylate complexes and the carboxylate shift - Implications for polymetalloprotein structure and function. New J. Chem. 1991; 15:417–430.

41. Grass G, Franke S, Taudte N, Nies DH, Kucharski LM, Maguire ME, Rensing C. The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. J. Bacteriol. 2005; 187:1604–1611. [PubMed: 15716430]

42. Mukhopadhyay S, Mandal SK, Bhaduri S, Armstrong WH. Manganese clusters with relevance to photosystem II. Chem. Rev. 2004; 104:3981–4026. [PubMed: 15352784]

43. Whittaker MM, Barynin VV, Igarashi T, Whittaker JW. Outer sphere mutagenesis of *Lactobacillus plantarum* manganese catalase disrupts the cluster core: Mechanistic implications. Eur. J. Biochem. 2003; 270:1102–1116. [PubMed: 12631270]

44. Atta M, Nordlund P, Aberg A, Eklund H, Fontecave M. Substitution of manganese for iron in ribonucleotide reductase from *Escherichia coli*: Spectroscopic and crystallographic characterization. J. Biol. Chem. 1992; 267:20682–20688. [PubMed: 1328209]

45. Jiang W, Yun D, Saleh L, Barr EW, Xing G, Hoffart LM, Maslak MA, Krebs C, Bollinger JMJ. A manganese(IV)/iron(III) cofactor in *Chlamydia trachomatis* ribonucleotide reductase. Science. 2007; 316:1188–1191. [PubMed: 17525338]

46. Högbom M. The manganese/iron-carboxylate proteins: what is what, where are they, and what can the sequences tell us? J. Biol. Inorg. Chem. 2010; 15:339–349. [PubMed: 20225400]

47. Naranuntarat A, Jensen LT, Pazicni S, Penner-Hahn JE, Culotta VC. The interaction of mitochondrial iron with manganese superoxide dismutase. J. Biol. Chem. 2009; 284:22633–22640. [PubMed: 19561359]

48. Archibald FS, Fridovich I. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. J. Bacteriol. 1981; 145:442–451. [PubMed: 6257639]

49. McNaughton RL, Reddi AR, Clement MHS, Sharma A, Barnese K, Rosenfeld L, Gralla EB, Valentine JS, Culotta VC, Hoffman BM. Probing in vivo Mn^{2+} speciation and oxidative stress resistance in yeast cells with electron-nuclear double resonance spectroscopy. Proc. Natl. Acad. Sci. U. S. A. 2010; 107:15335–15339. [PubMed: 20702768]
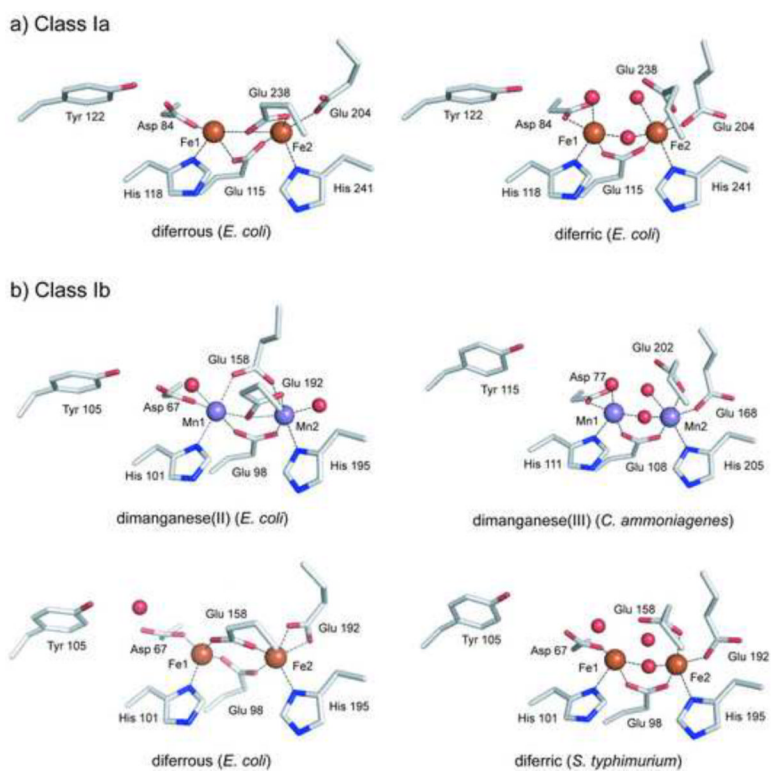
Figure 1.
Structures of the reduced and oxidized cofactors of the class Ia and Ib RNRs. Iron and manganese ions are depicted as brown and purple spheres, and solvent molecules are shown as red spheres. Generated using PyMOL from PDB files 1PIY (diferrous NrdB), 1MXR (diferric NrdB), 3N37 [dimanganese(II) NrdF], 3MJO [dimanganese(III) NrdF], 3N38 (diferrous NrdF), and 2R2F (diferric NrdF).
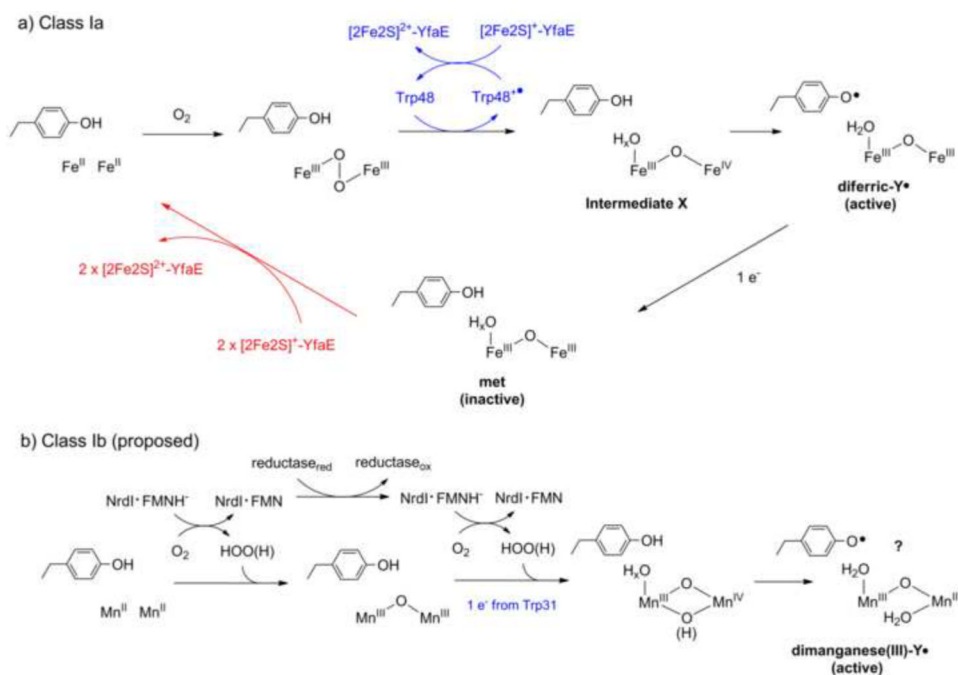
Figure 2.
Mechanisms of cofactor assembly (black) and maintenance (red) for class Ia and Ib RNRs. The intermediates shown in the class Ia mechanism have been spectroscopically characterized, whereas those in the class Ib mechanism are postulated based on analogy to the class Ia pathway. The structure of the dimanganese(III)-Y• cofactor shown is based on the crystal structure of *C. ammoniagenes* Mn$^{III}_2$-NrdF [6], but the possibility of photoreduction during data collection makes the identity of the bridging ligands in the active cofactor unclear. Blue text indicates the involvement of the "extra" electron in cofactor assembly.
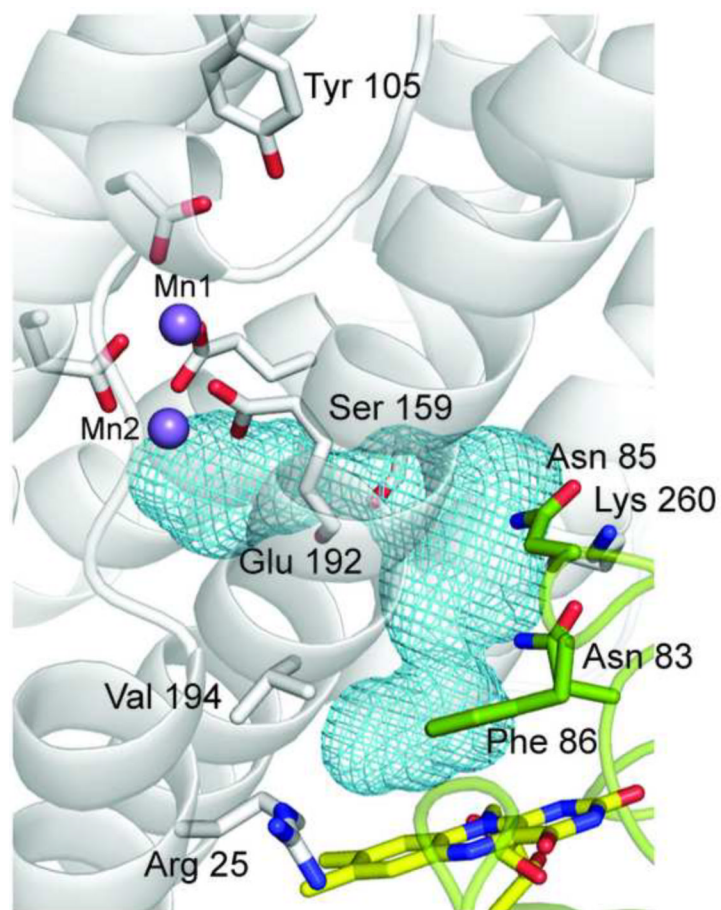
Figure 3.
The oxidant channel in the Mn^{II}_2-NrdF•NrdI$_{ox}$ complex. Binding of NrdI (green) to NrdF (gray) extends a hydrophilic channel (blue mesh) between the metal site and the flavin cofactor in NrdI (yellow sticks). Residues shown in sticks are highly or completely conserved in NrdIs and NrdFs. Reproduced from ref. [39].