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Metallation and mismetallation of iron and manganese proteins *in vitro* and *in vivo*: the class I ribonucleotide reductases as a case study

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Abstract

How cells ensure correct metallation of a given protein and whether a degree of promiscuity in metal binding has evolved are largely unanswered questions. In a classic case, iron- and manganese-dependent superoxide dismutases (SODs) catalyze the disproportionation of superoxide using highly similar protein scaffolds and nearly identical active sites. However, most of these enzymes are active with only one metal, although both metals can bind *in vitro* and *in vivo*. Iron(II) and manganese(II) bind weakly to most proteins and possess similar coordination preferences. Their distinct redox properties suggest that they are unlikely to be interchangeable in biological systems except when they function in Lewis acid catalytic roles, yet recent work suggests this is not always the case. This review summarizes the diversity of ways in which iron and manganese are substituted in similar or identical protein frameworks. As models, we discuss (1) enzymes, such as epimerases, thought to use Fe^{II} as a Lewis acid under normal growth conditions but which switch to Mn^{II} under oxidative stress; (2) extradiol dioxygenases, which have been found to use both Fe^{II} and Mn^{II}, the redox role of which in catalysis remains to be elucidated; (3) SODs, which use redox chemistry and are generally metal-specific; and (4) the class I ribonucleotide reductases (RNRs), which have evolved unique biosynthetic pathways to control metallation. The primary focus is the class Ib RNRs, which can catalyze formation of a stable radical on a tyrosine residue in their β 2 subunits using either a di-iron or a recently characterized dimanganese cofactor. The physiological roles of enzymes that can switch between iron and manganese cofactors are discussed, as are insights obtained from the studies of many groups regarding iron and manganese homeostasis and the divergent and convergent strategies organisms use for control of protein metallation. We propose that, in many of the systems discussed, “discrimination” between metals is not performed by the protein itself, but it is instead determined by the environment in which the protein is expressed.

Introduction

Mismetallation *in vitro*

Biochemical work often relies on the overexpression of a protein of interest in a heterologous host, usually *Escherichia coli*, grown under “standard” laboratory conditions in iron-rich media. When the protein requires a metal for activity, insertion of the correct metal depends on the protein’s affinity for and selectivity between metals available in the cell

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during or after folding, the cellular concentrations of the metals and the protein,^{1–3} and in some cases, the presence of cofactor biosynthetic machinery specific to the metal and/or protein. Proteins overexpressed are often isolated with the incorrect metal (mismetallated)⁴ or, if the biosynthetic machinery is not up-regulated or if the metal is weakly bound, they can be isolated predominantly in their apo form (no metal bound). Iron–sulfur clusters,^{5,6} the complex cofactors of nitrogenases⁷ and hydrogenases,^{8,9} hemes,¹⁰ cobalamins,¹¹ and the dimanganese(III)-tyrosyl radical ($\text{Mn}^{\text{III}}_2\text{-Y}^*$) cofactor^{12,13} of the class Ib ribonucleotide reductases (RNRs) are a few examples of cofactors demonstrated to require biosynthetic machinery for assembly.

Apo-proteins are catalytically inactive. More insidiously, a mismetallated protein may retain some biological activity. Because, in general, proteins are not isolated from their endogenous organisms under endogenous levels of expression, their actual catalytic activity is not known. There are myriad examples in which a correlation of metal with catalytic activity has been elusive: peptide deformylase,^{14,15} calprotectin,¹⁶ particulate methane monooxygenase (MMO),^{17,18} the arylamine-*N*-oxygenase AurF,^{19,20} some Mn- and Fe-superoxide dismutases (SODs),^{21,22} and the class Ib¹² and Ic²³ RNRs. Furthermore, many metallocofactors can self-assemble *in vitro* with some degree of success from apo-protein and a metal source, even when biosynthetic pathways are utilized *in vivo*. Thus, comparison of activities of apo-protein reconstituted *in vitro* with various metals provides insight into candidate metals for *in vivo* relevance; however, the metal that confers the highest activity is not necessarily the physiologically relevant cofactor. Ultimately, understanding the cellular context in which a given protein is expressed is crucial to the correct assignment of the cofactor.

In this review, we consider these general issues in the specific context of the interplay of Mn and Fe in biological systems. Of the first row transition metals used commonly by biology (Mn, Fe, Co, Ni, Cu, and Zn), Mn^{II} and Fe^{II} generally bind most weakly to proteins and prefer similar coordination environments, making it difficult for proteins to distinguish between them on the basis of structure alone. At the same time, the midpoint potentials for the +III/+II couples of the hexaquo Mn^{II} and Fe^{II} ions are 1.5 and 0.8 V (*vs.* NHE),²⁴ respectively, making the redox chemistry of the two metals far from interchangeable. A growing body of evidence suggests that aerobic organisms have exploited the similar and dissimilar properties of these two metals in ingenious ways to protect themselves from oxidative damage and iron deprivation, frequently encountered in their normal habitats. In some cases, the substitution of Fe with Mn can occur in the same polypeptide; in others, separate Mn- and Fe-dependent enzymes are used. In each case, the Fe and Mn binding sites are nearly identical (Fig. 1). Here we examine four increasingly complex examples of this ingenuity (reactions shown in Fig. 2): (1) enzymes that use Fe^{II} or Mn^{II} interchangeably as a Lewis acid catalyst;²⁵ (2) Fe^{II} - and Mn^{II} -containing extradiol dioxygenases,^{26,27} such as homoprotocatechuate 2,3-dioxygenase (HPCD), which catalyze a redox reaction but for which the involvement of the metal ions in a redox role is unresolved; (3) Fe- and Mn-SODs, which cycle their mononuclear metal cofactors between +II and +III states to disproportionate $\text{O}_2^{\bullet-}$;²⁸ and (4) the class I ribonucleotide reductases (RNRs), which use diiron, dimanganese, and manganese–iron cofactors^{29,30} to generate a stable protein- or metal-based radical cofactor essential for deoxynucleotide formation in all eukaryotes and most aerobic prokaryotes. We focus mainly on the class I RNRs because they beautifully illustrate the challenges of identifying a physiological metallocofactor, the utility of parallel *in vitro* and *in vivo* studies in this determination, and the adaptations Nature must make to accommodate the chemical differences between manganese and iron in redox reactions.

By consideration of these specific systems, we hope to provide general insight into how an experimentalist identifies the physiologically relevant cofactor of a metalloprotein and how

this information sheds light on the mechanism(s) by which the organism ensures that protein's correct metallation. As will become apparent, these studies are in their relative infancy, and much more data needs to be collected before a general model can emerge. However, in our view, the results to date can be accounted for most simply, in the case of prokaryotes at least, by a model in which a protein's ability to discriminate between iron and manganese is chiefly dictated by metal availability, rather than a structural preference of that protein for Fe^{II} or Mn^{II}. Metal availability, in turn, is determined by how the organism tunes its overall metal homeostatic machinery. Therefore, it is likely that the metallation of many of these enzymes that can be activated with either Mn or Fe will be chiefly governed within a given organism by the conditions in which the protein is expressed. Whether this is the only, or the primary, mechanism remains to be determined.

Mismetallation *in vivo*

Mismetallation is not only a problem when proteins are present at non-physiological levels in non-physiological growth conditions; it is also a challenge for organisms during routine growth. While some "mismetallated" enzymes may retain activity, others are inactive. Cellular metal ion homeostasis must be managed carefully because cells use a variety of metals, because certain transition metals (chiefly Fe^{II} and Cu^I) can react with O₂ to produce reductive metabolites of O₂ that are damaging to cells,³⁷ and because a given protein can bind many metals and the tightest binding metal is often not the "correct" one required for function. To ensure that the correct metal is inserted into a protein and, perhaps more importantly, to prevent the incorrect metal from binding, cells express metallochaperones³⁸ for some of the tightest binding metals, Cu^I,^{39,40} Co^{II},^{41,42} and Ni^{II}.⁴³ For example, the greater than femtomolar (10⁻¹⁵ M) affinities of copper enzymes and chaperones for Cu^I make the importance of having copper chaperones clear and their identification relatively facile. Because Fe^{II} and Mn^{II} are typically the weakest binders to proteins of the biologically used first row metals,⁴⁴ however, the existence of chaperones for these metals is less certain.

Some prokaryotes, like *E. coli*, accumulate μM to mM levels of total iron, much of it in a "labile iron pool," weakly bound to proteins or small molecules.⁴⁵ A major challenge is to quantify⁴⁶ and to determine the composition of this pool.⁴⁷ The deleterious chemistry of the reactions of Fe^{II} with O₂ and H₂O₂ (Fenton chemistry) suggests that sequestering iron in a less reactive form by a protein chaperone or a small molecule would be beneficial to the cell. However, whether such proteins or small molecules are involved in transfer of iron to enzymes through specific interactions is an active area of investigation. Putative iron chaperones have been reported in eukaryotic systems to transfer of iron into ferritin⁴⁸ and prolyl hydroxylases⁴⁹ (PCBP1 and 2)⁵⁰ and in iron-sulfur cluster and non-heme diiron cluster assembly (frataxin⁵¹ and Grx 3/4⁴⁷). The dissociation constants of PCBP1 and frataxin for Fe^{II} have been measured to be ~5 μM.^{48,51} With such weak binding, it is unclear whether these putative chaperones are essentially glorified small-molecule ligands of a "labile" iron pool or if they are designed to deliver iron specifically to defined protein targets. In the case of Mn^{II}, studies have also indicated the existence of μM to mM labile Mn^{II} pools^{52,53} in organisms from lactic acid bacteria to yeast, largely bound to abundant ligands like phosphate (*K*_d for Mn^{II} binding is 0.5 mM⁵⁴) and pyrophosphate,⁵³ but no chaperone proteins have been identified to date. Indeed, perhaps none are necessary as, in addition to its weak binding to biological ligand sets, the redox chemistry of Mn^{II} when bound to these biological small molecule ligands can counteract oxidative stress (*vide infra*),⁵⁴ in contrast with Fe^{II}.³⁷

The weak binding of Mn^{II} and Fe^{II} to enzymes and metal-sensing transcription factors is reflected in Table 1. These data highlight two points: first, few *K*_ds are known, and second, of those that are, the affinities are generally fairly low and in the range of reported cellular concentrations of the metals (the exception is MnSOD, which will be discussed below).

Thus correct metallation may be determined by the relative affinities of the two metals for the protein and the “differential bioavailability” of the two metals in a given growth condition. This model has been proposed by Culotta and coworkers¹ to explain how *S. cerevisiae* MnSOD is correctly metallated even in the presence of high, but sequestered, levels of iron in the mitochondrial matrix. When iron–sulfur cluster assembly is disrupted or when cells are starved for Mn, however, the protein is mismetallated with Fe.^{1,55} In a variation on this theme, correct metallation of a Mn^{II}-containing periplasmic protein is ensured by its folding in the cytosol, where available Mn^{II} levels are much higher than those of Cu and Zn, whereas a related Cu^{II}-dependent protein with identical metal ligands folds in the periplasm, where Cu is more bioavailable.³ As argued above, given the generally weak binding of Mn^{II} and Fe^{II} to proteins, protein chaperones would reasonably also bind these metals weakly and thus not dramatically affect their bioavailable supply any more than a small molecule would. Thus, whether or not Fe^{II} and Mn^{II} are sequestered by protein chaperones in prokaryotes, the model of differential bioavailability may be sufficient to account for the metallation patterns of the prokaryotic Mn- and Fe-dependent proteins we now examine, and possibly in general in proteins that do not require more complex cofactors containing unique ligands, such as nitrogenases⁷ and hydrogenases.^{8,9}

Interplay of Mn and Fe in biological systems

Replacement of Fe^{II} with Mn^{II} in non-redox-active enzymes in response to oxidative stress

In a first example of the interchangeability of iron and manganese in aerobes, we focus on enzymes in which divalent metal ions play roles as Lewis acid catalysts. This issue has recently been studied by the Imlay lab in ribulose 5-phosphate 3-epimerase, peptide deformylase, threonine dehydrogenase, and cytosine deaminase. These enzymes were targeted for study as they show no commonality in mechanism aside from the use of divalent metal ions as Lewis acid catalysts. Furthermore, the enzymes are active *in vitro* with multiple metal ions and the identity of the metal ion(s) used by these enzymes *in vivo* has been controversial. Based on genetic and biochemical studies, the Imlay lab has suggested that these enzymes in *E. coli* typically use Fe^{II} as a cofactor, which is replaced by Mn^{II} under oxidative stress conditions.

Organisms have evolved specialized responses to defend against oxidative stress agents, such as H₂O₂ and O₂^{•-}.³⁷ These species originate intracellularly from normal aerobic metabolism as well as extracellularly, such as H₂O₂ produced by lactic acid bacteria and streptococci, or O₂^{•-} and H₂O₂ produced by phagosomal NADPH oxidase, in both cases used as an antimicrobial defense. The amounts of these species that can be tolerated varies depending on the organism. In *E. coli*, the H₂O₂-responsive transcriptional regulator OxyR is activated at ~0.2 μM intracellular H₂O₂,⁶⁹ and levels of 0.5–1 μM have been shown to be toxic to certain enzymatic pathways, such as iron–sulfur cluster assembly.^{68,70} During H₂O₂-induced stress, OxyR mediates up-regulation of the Mn^{II} importer MntH, stimulating Mn^{II} uptake,⁷¹ and of the ferritin-like protein Dps, which uses H₂O₂ to sequester Fe^{II} (Fig. 3).^{72,73} Imlay and coworkers have proposed that this response to decrease bioavailable Fe^{II} and to increase bioavailable Mn^{II} (from ~15 to 150 μM)⁷¹ represents a strategy to replace Fe^{II} in the active site of some enzymes with Mn^{II}. This switch is proposed to protect the enzymes from self-inactivation by any number of mechanisms that involve Fe^{II} oxidation by H₂O₂, leading to Fe^{III} dissociation and oxidative damage to the protein.

Recently, ribulose 5-phosphate 3-epimerase (Rpe, Fig. 2), an enzyme in the pentose phosphate pathway, was identified by Imlay and coworkers as an Fe^{II}-utilizing enzyme under “normal” growth conditions that can self-inactivate under oxidative stress, accompanied by iron loss.²⁵ Inactivation was prevented by increasing the levels of Mn^{II} in the culture media, presumably by replacing Fe^{II} with Mn^{II} in the epimerase’s active site.

Unfortunately, the weak binding of both Mn^{II} and Fe^{II} to Rpe prevented isolation of metallated protein and thus the endogenous metal cofactor could not be identified directly. Instead, the determination relied on activity assays in crude extracts of oxidatively stressed cells coupled to *in vitro* assays of recombinant protein metallated with Fe^{II} , Mn^{II} , Co^{II} , or Zn^{II} (Rpe copurifies with Zn^{II} when overexpressed in *E. coli*⁷⁴).

Further evidence for the proposed metal swap was obtained for three other Lewis acid enzymes, peptide deformylase, threonine dehydrogenase and cytosine deaminase (CDA).¹⁵ As with Rpe, H_2O_2 inactivates these enzymes in crude extracts, but only the Fe^{II} -bound recombinant enzymes were inactivated *in vitro*. For the first two enzymes, data suggested modification of the metal's Cys ligand, and the inactivation was reversible upon addition of reductant and Fe^{II} . In CDA, in which the metal is not ligated by a Cys, inactivation was only partially reversible. To test whether Mn^{II} was able to prevent inactivation of these enzymes *in vivo*, ΔmntH and Δdps mutants were constructed in an *E. coli* strain that was constitutively H_2O_2 -stressed. All three enzymes were inactivated, recoverable by addition of Mn^{II} to the culture media except in the case of CDA. Thus the authors suggested that metal swaps represent a general strategy by *E. coli* to stave off oxidative damage to enzymes that typically use iron cofactors. To explain the observation of partial irreversible inactivation of CDA specifically, it was proposed that Cys ligands to putative Fe^{II} cofactors prevent more serious enzyme damage from reaction of Fe^{II} with H_2O_2 . However, cysteine serving as a ligand to Mn^{II} in a biological system would be unprecedented, to our knowledge. In fact, sulfur (in the form of the thioether of Met) appears to be important for *in vivo* discrimination of Fe^{II} over Mn^{II} by the metalloregulator DtxR from *Corynebacterium diphtheriae*.⁷⁵ Unfortunately, direct observation of Mn^{II} bound to the proteins has been elusive, due its weak binding. Also unresolved is whether the putative Mn^{II} -bound forms of the proteins are active *in vivo* or just protective until the end of oxidative stress, and whether these proteins are typically Mn^{II} enzymes in other organisms for which intracellular manganese is present in higher concentrations during normal growth (*vide infra*). Further in-cell methods are necessary to address these important but difficult questions.

Fe^{II} - and Mn^{II} -dependent extradiol dioxygenases

Another example of successful metal substitution of Fe^{II} and Mn^{II} is found in the extradiol dioxygenases, such as the homoprotocatechuate (HPCA) 2,3-dioxygenases (HPCDs). Extradiol dioxygenases use a divalent metal site and O_2 to cleave the ring of a catecholic aromatic substrate adjacent to its two hydroxyl groups (Fig. 2B).²⁷ In these enzymes, the metal ion binds to a 2-His-1-carboxylate facial motif, leaving three open coordination sites, two of which are occupied by solvent ligands in the resting form of the enzyme.^{76,77} The catecholic substrate binds to the metal, occupying two coordination sites, releasing the bound solvent molecules and leaving one site open for O_2 binding (Fig. 1A and 2B).^{78–80} Most of the extradiol dioxygenases purified to date are assigned as Fe^{II} dependent, but a few are Mn^{II} dependent. HPCDs from *Arthrobacter globiformis* CM-2 (MndD)⁸¹ and from *Brevibacillus* (formerly *Bacillus*) *brevis*,⁸² and BphC from *Bacillus* sp. JF8⁸³ have been purified from their native organisms and found to contain Mn^{II} . *Bacillus* sp. JF8 has also been proposed to encode another Mn^{II} -dependent dioxygenase as well as an Fe^{II} -dependent one,⁸⁴ although the metal selectivities of these proteins have only been examined in recombinant systems. A dioxygenase from *Klebsiella pneumoniae* is claimed to be Mg^{II} -dependent.⁸⁵ While a detailed analysis of the metal requirements of the purified extradiol dioxygenases is beyond the scope of this review, it is important to note that not all of the “ Fe^{II} -dependent” enzymes have been rigorously demonstrated to be iron enzymes in their physiological growth conditions. For example, although purified from its native organism, the HPCD from *Brevibacterium fuscum*⁸⁶ was purified from growth conditions in which the minimal medium was supplemented with 50 μM Fe^{II} and no other metals.

To probe the origin of metal specificity and mechanism of the Fe- and Mn-dependent extradiol dioxygenases, the Lipscomb and Que labs have carried out in-depth metal substitution experiments with Fe^{II}, Mn^{II}, and Co^{II} using the *B. fuscum* (Fe–HPCD) and *A. globiformis* (Mn–MndD) enzymes.⁸⁷ Their results have shown that these two enzymes³¹ and their “mismetallated” forms all have nearly identical catalytic activities and active site geometric (Fig. 1A) and electronic structures, despite the disparity in +III/+II reduction potentials of hexaaquo Co, Mn, and Fe (1.9 V vs. 1.5 V vs. 0.8 V; these values for the enzyme-bound metals have not been determined).⁸⁸ These similarities initially led to the proposal that the mechanism may not involve a metal-based redox reaction.²⁶ Subsequent mechanistic studies using rapid kinetics methods reported small quantities (5% of total Mn) of a Mn^{III}-radical intermediate proposed to be Mn^{III}-superoxide⁸⁹ in the Mn^{II}-substituted *B. fuscum* HPCD with HPCA as substrate. In a similar set of experiments using an Fe–HPCD with the active site acid–base catalyst His mutated to Asn, which slows the reaction, and with 4-nitrocatechol (4-NC), a very slow substrate due to the presence of the electron-withdrawing nitro group, a Fe^{III}-superoxo intermediate (~10% of total Fe) was reported.^{90,91} However, the use of the mutant and unnatural substrate together abolishes ring cleavage; an important issue is therefore whether this intermediate, although early in the reaction, is “on-pathway” for the wt enzyme with the natural substrate. Nevertheless, these results led to a revised model in which the M^{III}-superoxo intermediate is rapidly reduced by the catechol substrate to form a reactive M^{II}-(semiquinone)-superoxo species. Thus it is now proposed that the metal ion serves as a “conduit” for electron transfer between substrate and O₂ and that the metal ion’s redox potential is of little importance for overall enzyme function as long as the M^{III}-superoxo can be accessed in a reasonable amount of time.⁸⁸ In fact, although a higher metal reduction potential would slow formation of the putative M^{III}-superoxo intermediate, it would also favor electron transfer from the substrate to the metal, perhaps accounting for the similar activities of the Co^{II}-, Mn^{II}-, and Fe^{II}-substituted HPCDs.⁸⁸ How much the protein perturbs the reduction potentials of the bound metal ions in these systems is at present unknown, but it seems likely that the electron-rich nature of the substrate is critical for facilitating formation of a M^{III}-superoxo species with Mn–HPCD to compensate for the high metal reduction potential. It will be interesting to know if 4-NC can serve as a substrate for the Mn^{II} form of the enzyme, or whether its higher reduction potentials precludes this reaction. Such studies could help resolve the question of whether the metal is truly redox active in these enzymes.

Although the interchangeability of metal ions in the active sites of these extradiol dioxygenases has been established *in vitro*, the question of whether this interchangeability is relevant *in vivo* as well remains unresolved. As we will see below, some of the organisms that possess putative Mn^{II}-dependent dioxygenases (*Arthrobacter* and *Brevibacillus*) also possess class Ib RNRs that are likely Mn-dependent as well. This correlation may suggest that, while both enzymes could theoretically be metallated with either Mn or Fe *in vivo*, the overall Mn^{II} versus Fe^{II} concentrations in these organisms are the primary determinants of “metal selectivity” in these enzymes and are responsible for the observed metal dependences.

Fe– and Mn–SODs

In the case where the Fe and Mn ions must undergo changes in redox state during catalysis, the consequences of mismetallation change. The classic example is the Fe– and Mn–SODs, which disproportionate superoxide to O₂ and H₂O₂, cycling between their + II and + III states (Fig. 2C). These enzymes are structurally homologous, and their active sites are identical in the first coordination sphere (Fig. 1B). While there have been a few reports of “cambialistic” SODs that are active with either metal,⁹² most Mn– and Fe–SODs, like those of *E. coli*, display significant activity only with one metal. As recently summarized by Miller,²⁸ each

Mn- or Fe-SOD tunes the redox potentials of its “correct” metal ion to fall between the potentials of the two half-reactions (0.89 V for $O_2^{\bullet-}$ reduction to H_2O_2 , -0.16 V for reduction of O_2 to $O_2^{\bullet-}$).⁹³ Because of the higher reduction potential of Mn^{III} than Fe^{III} , the Mn-SOD protein must depress the reduction potentials of its bound metal much more than Fe-SOD; therefore substitution of Fe in a Mn-SOD would generate a protein only able to reduce $O_2^{\bullet-}$.⁹⁴ SODs modulate the potentials of their respective metal ions by differential tuning of hydrogen-bonding interactions between solvent ligands to the metals and the protein scaffold.^{28,94,95} As with the extradiol dioxygenases, there is no marked preference for Fe over Mn binding or *vice versa*. For example, given that the cellular Mn^{II} and Fe^{II} concentrations are much higher than the K_{ds} for *E. coli* MnSOD given in Table 1 and that metal binding has been shown to be irreversible,² the enzyme’s affinities for Mn^{II} and Fe^{II} are similar enough that MnSOD is metallated with both Mn and Fe in wildtype *E. coli* grown in rich medium.²¹ While this may seem inefficient, *E. coli* also encodes an Fe-dependent SOD; furthermore, in oxidative stress and Fe deficiency, MnSOD is induced and becomes preferentially loaded with Mn,^{96,97} probably as a result of both lower levels of free iron (through its oxidation to Fe^{III}) and higher levels of Mn^{II} due to increased Mn import (Fig. 3).

The complexity of metallation of SODs with Fe and Mn is also illustrated by recent studies of the enzymes from the pathogen *Bacillus anthracis*.²² Waldron and coworkers grew *B. anthracis* in rich media, lysed cells anaerobically, separated the lysate by two-dimensional liquid chromatography, and analyzed Fe and Mn contents in the resulting chromatography fractions. While a limitation of this method is that only proteins that tightly bind their metal cofactors can be identified, SODs fall into this category. The researchers found that *B. anthracis* contains one SOD primarily loaded with Mn (SodA1, with 13% containing Fe) and another (SodA2) exclusively loaded with Fe. The two SODs were also cloned and overexpressed in *E. coli*, both copurifying mostly with Fe. Whereas Fe could be removed from SodA1 and the protein could be reconstituted with Mn^{II} to increase activity 30-fold, SodA2 could only be loaded and activated with Fe, unusual as most apo-SODs can be mismetallated *in vitro* by the same protocol as they are metallated. The observation that SodA1 is primarily loaded with Mn in *B. anthracis* even in rich media and loaded primarily with Fe when overexpressed in *E. coli* suggests that Mn and Fe pools are not as imbalanced in *B. anthracis* as they are in *E. coli*, or that metallation of SodA1 with Mn in *B. anthracis* requires a Mn^{II} chaperone.

In support of the former option, typical Mn^{II} levels are likely higher in *B. anthracis* than in *E. coli* (15 μM in normal growth conditions) based on the affinity of the Mn^{II} -dependent transcriptional regulator AntR for Mn^{II} .⁶³ Metalloregulatory proteins translate information about intracellular metal concentrations into a transcriptional response through differences in affinity for their DNA binding sites in the apo and metal-bound forms; therefore, their K_{ds} for their cognate metals should approximate the typical intracellular “free” concentrations of those metals.⁹⁸ For example, the concentrations of “free” Cu and Zn ions in the cell are vanishingly low,^{45,99} and the sensors for these ions in *E. coli* have K_{ds} in the 10^{-15} – 10^{-21} M range.^{100,101} AntR binds two Mn^{II} per monomer in a positively cooperative process with K_{ds} of 210 and 17 μM ,⁶³ suggesting “free” Mn^{II} levels in the 100–200 μM range in *B. anthracis*. Thus, while metallation of SodA1 may be driven by high Mn levels, SodA2 may need to exclude Mn^{II} binding, as it does *in vitro*, by a yet to be determined mechanism in order to be metallated with Fe *in vivo*.

Interestingly, unlike *E. coli*,⁷¹ oxidative stress does not appear to induce Mn^{II} import in wild-type *B. anthracis*;^{22,102} however, growth of *B. anthracis* is impaired in *sodA1* and *sodA2* mutants cultured in Mn-depleted media.¹⁰² These results, together with the affinity of AntR for Mn^{II} , may suggest that pools of Mn^{II} are important in normal growth for this

organism and are essential in the absence of enzymes for oxidative defense. Such a cytoprotective function for Mn^{II} pools is reminiscent of the observation that some bacteria have done away with SOD enzymes altogether, instead relying on non-enzymatic disproportionation of $\text{O}_2^{\bullet-}$ by cellular Mn^{II} . The utility of “free” Mn for oxidative defense has been supported by *in vitro*^{54,103} and *in vivo*⁵³ evidence. Mn^{II} -phosphate complexes, for example, can catalyze disproportionation of $\text{O}_2^{\bullet-}$ at a rate sufficient to manage $\text{O}_2^{\bullet-}$ stress at biologically accessible concentrations of Mn^{II} and phosphate. This chemistry has also been proposed to be exploited by pathogens, for which manganese transporters have been shown in a number of cases to be key for virulence.^{104,105} These data indicating Mn^{II} accumulation suggest that the strategy of utilizing Mn^{II} to help combat oxidative stress is widely conserved; even organisms like *E. coli*, for which Mn^{II} plays an apparently minor role in growth in iron-rich media, revert to this strategy in conditions of oxidative stress, as illustrated by the example of Rpe described above.^{25,71}

Class I ribonucleotide reductases

The most complex substitution of Mn and Fe observed so far is found in the class I RNRs. RNRs catalyze the reduction of nucleoside 5'-di- or triphosphates (NDPs or NTPs) and constitute the only pathway for de novo synthesis of deoxy-nucleotides in all organisms.^{106,107} Nucleotide reduction is initiated by abstraction of the 3' hydrogen atom of the NDP substrate by a thiyl radical¹⁰⁸ generated transiently in the active site. After loss of the substrate 2'-OH as water and reduction concomitant with oxidation of cysteine residues to a disulfide, the 3' hydrogen atom is returned to C3', regenerating the thiyl radical (Fig. 2D).¹⁰⁹ Nature has devised three ways of reversible formation of the thiyl radical (Fig. 4). In the class III RNRs, expressed only under strictly anaerobic conditions, the cysteine is oxidized directly by a stable glycyl radical in turn generated by a radical-S-adenosylmethionine activating enzyme.¹¹⁰ In the O_2 -independent class II RNRs, a 5'-deoxy-adenosyl radical formed by homolysis of the cobalt-carbon bond of adenosylcobalamin oxidizes the cysteine.¹¹⁰ In the O_2 -dependent class I RNRs, the active site cysteine in the $\alpha 2$ subunit is oxidized by a metallocofactor 35 Å away in the $\beta 2$ subunit of the enzyme by a proton-coupled electron transfer pathway composed of conserved aromatic amino acid residues.¹¹¹⁻¹¹³ The $\beta 2$ subunit is homologous to other, mostly diiron proteins of the ferritin superfamily such as methane monooxygenase, Δ -9 desaturase, Dps, ferritin, and the Mn-dependent catalases.¹¹⁴⁻¹¹⁶ As a result of several recent discoveries, the class I RNRs have been divided into three subclasses – Ia, Ib, and Ic – based on identity of the metallocofactor (Fig. 4).³⁰ Phylogeny, details of allosteric regulation, and requirements for unique accessory factors for metallocofactor assembly also help to distinguish these subclasses.

Just five years ago, the role of a manganese-containing metallocofactor in any class I RNR was controversial; the class Ia, Ib,^{107,117} and Ic^{118,119} enzymes were all generally believed to be di-iron proteins *in vivo*. The class Ia RNRs are found in all eukaryotes and some prokaryotes, such as *E. coli*.¹²⁰ The *E. coli* class Ia RNR was the first to be purified and is the best characterized system to date. Classic experiments in the 1970s identified the source of an EPR signal in preparations of the $\beta 2$ subunit (NrdB) as a tyrosyl radical (Y^{\bullet}),¹²¹⁻¹²³ which was subsequently localized to a tyrosine residue adjacent to a μ -oxo-bridged diferric cluster (Fig. 1C).¹²⁴ This Y^{\bullet} was the first protein radical observed in biology. The Y^{\bullet} is absolutely essential for RNR activity and activity scales with Y^{\bullet} content.¹²⁵ *In vitro* experiments carried out by Atkin *et al.*¹²⁶ demonstrated that the diferric- Y^{\bullet} cofactor could self-assemble from apo-NrdB, Fe^{II} , and O_2 . Numerous studies using an arsenal of biophysical methods support the general mechanism of diferric- Y^{\bullet} cofactor assembly shown in Fig. 5A.¹²⁷⁻¹³¹ The “extra” electron required can be derived *in vitro* from Fe^{II} , ascorbate,

or thiols,¹²⁷ but *in vivo* in *E. coli*, it is proposed that the [2Fe2S]-ferredoxin YfaE is the source of this reducing equivalent (*vide infra*).¹³²

The class Ib RNRs – found only in prokaryotes, including numerous pathogens such as *Mycobacterium tuberculosis*, *B. anthracis*, and *Staphylococcus aureus*, in addition to *E. coli* – exhibit low sequence identity with class Ia RNRs (~30% for *E. coli* class Ia and Ib RNRs). Nevertheless, the class Ib RNR α 2 (NrdE)¹³³ and β 2 (NrdF)¹³⁴ subunits are structurally homologous to the corresponding class Ia subunits and possess identical metal-binding residues (Fig. 1C). The second coordination spheres are subtly different (discussed in detail in ref. 35 and 134). Evidence primarily from the Auling and Follmann laboratories in the 1980s and 1990s suggested that corynebacterial NrdFs bind Mn inside the cell.^{135,136} However, definitive identification of the cofactor was hampered by the inherent instability of Y[•] and consequent very low specific activity due to the extensive period required for purification. No Y[•] was detected and hence its relationship to Mn content could not be correlated. At the same time, the diferric-Y[•] cofactor had been shown by a number of groups to self-assemble from apo-NrdF, Fe^{II}, and O₂, just as in class Ia RNRs, and was catalytically active. By contrast, Y[•] could not be detected in efforts to self-assemble the cluster from Mn^{II} with O₂.¹¹⁷ Therefore, the diferric-Y[•] was assumed by some^{117,137} to be the actual cofactor of the class Ib RNRs *in vivo*.

Recently, however, studies from our lab¹² and the Auling and Lubitz labs¹³ demonstrated definitively that a Mn^{III} γ -Y[•] cofactor could be generated *in vitro* and *in vivo*, respectively. In the former case, this process was shown to require^{12,138} the presence of a conserved accessory protein, NrdI^{139,140} (Fig. 5B). The physiological relevance of the dimanganese cofactor has recently been demonstrated under a variety of growth conditions in *Corynebacterium ammoniagenes*,¹³ *E. coli*,^{66,141} and *B. subtilis*.¹⁴² The observation that both Mn^{III} γ -Y[•] and Fe^{III} γ -Y[•] cofactors in the class Ib RNRs can initiate nucleotide reduction *in vitro*, however, raises the issue as to whether certain growth conditions will favor the formation of the latter cofactor *in vivo* as well.

The class Ic RNRs (Fig. 5C), identified by sequence analysis in a limited number (~50 as of 2010)¹⁴³ of prokaryotes, have replaced the conserved tyrosine residue adjacent to the metal cluster of the class Ia and Ib RNRs with a non-oxidizable residue such as Phe.¹¹⁸ Therefore, the oxidizing equivalent necessary for thyl radical formation must be stored in the metal cluster itself. Early experiments^{118,119} on the *Chlamydia trachomatis* RNR, the only class Ic RNR studied to date, suggested that a Fe^{III}Fe^{IV} cofactor, the analogue to the tyrosine-oxidizing intermediate of the class Ia RNRs (X, Fig. 5A), was active in nucleotide reduction. However, subsequent work of the Bollinger–Krebs lab^{23,144} established that the protein could be activated with an unprecedented Mn^{IV}Fe^{III} cofactor by the general mechanism shown in Fig. 5C and suggested that the Fe^{III}Fe^{IV} cofactor is inactive. The history of the discovery of the *C. trachomatis* class Ic RNR's Mn^{IV}Fe^{III} cofactor has been recently reviewed^{29,30,145,146} and will not be described in detail here. Important unresolved issues remain, however, including whether the Fe^{III}Fe^{IV} form is totally inactive²³ or can catalyze a single turnover^{147,148} and the identity of the physiological cofactor. Because the *in vivo* metallation state of class Ic RNRs has not been established and because the class Ib RNRs are the only RNRs that can catalyze multiple turnovers using two different cofactors, we primarily restrict our remaining discussion to the class Ib RNR.

Determination of a physiological metallocofactor: the class Ib RNRs

In 1988 Auling and Follman isolated a manganese-containing RNR from *C. ammoniagenes* that had very low enzymatic activity and no detectable Y[•].¹³⁶ Based on their results and the biology of *C. ammoniagenes*,^{135,149,150} they proposed that there would be a manganese, tyrosyl radical-dependent RNR. However, a controversy over the identity of the class Ib

RNR metallocofactor has persisted until recently, due to inability to detect the proposed Y^* . The history has been recently recounted^{29,30,151} with the important milestones summarized in Table 2. Briefly, the incorporation of active diferric- Y^* cofactor into NrdFs during their overexpression in *E. coli* in rich media,¹⁵² the ability to self-assemble an active diferric- Y^* cofactor *in vitro* as in the class Ia RNRs,¹¹⁷ the inability to self-assemble an active manganese- Y^* cofactor under a range of conditions,^{12,117} and the low activity associated with the purified manganese-containing RNRs,^{136,153,154} led several groups to favor the importance of the iron cofactor *in vivo* and to propose that the activity of the manganese-containing RNR was associated with small amounts of active diferric- Y^* cofactor. However, in late 2009, Auling and coworkers published evidence strongly suggesting that NrdF from *Corynebacterium glutamicum*, closely related to the *C. ammoniagenes* NrdF, contained a manganese- Y^* cofactor.¹⁵⁵

The role of NrdI

The definitive link between the presence of manganese in NrdF, Y^* , and nucleotide reduction activity was provided when we demonstrated for the first time that an active Mn^{III}_2 - Y^* cofactor could be assembled *in vitro*.¹² The key was the consideration that a biosynthetic pathway would be essential for formation of the metallocofactor, and the missing link was provided by NrdI. In 2008 we had shown that *nrdI*, a universally conserved gene in organisms containing class Ib RNRs and often located on an operon with *nrdE* and *nrdF*,¹²⁰ encodes a flavodoxin-like protein that interacts with NrdF.¹⁴⁰ When apo-NrdF was incubated with Mn^{II} and reduced NrdI (NrdI_{hq}) and then exposed to O_2 , Y^* coupled to a dimanganese cluster, proposed to be Mn^{III}_2 based on UV-vis and EPR evidence and mechanistic considerations, was generated.¹² Further experiments suggested that NrdI_{hq} bound to NrdF and, when reacted with O_2 , produced an oxidant that was channeled to the metal cluster in NrdF. This proposal was supported by crystallization of *E. coli* NrdI- Mn^{II}_2 -NrdF complexes,¹³⁸ which revealed a channel connecting the FMN cofactor in NrdI to Mn_2 in the Mn^{II}_2 site in NrdF (Fig. 6). The hydrophilicity of the channel and the nearly completely conserved residues from NrdF and NrdI that line the channel support a hydrophilic oxidant such as HOO(H) or $O_2^{\cdot-}$,³⁰ the identity of the oxidant is unresolved. Based on structure and sequence alignments, this channel is located in the same area as the previously proposed access route for O_2 to the diferrous site of the class Ia RNRs.^{156,157} Interestingly, NrdI_{hq} is unable to activate the class Ia RNR loaded with Mn^{II}_2 cluster, despite the structural similarity of the class Ia and Ib diferrous and dimanganese(II) sites.^{35,141,158} Thus the key to Mn^{III}_2 - Y^* cofactor assembly in the class Ib, but not class Ia, RNR is that Mn^{II}_2 -NrdF forms a complex with NrdI and a unique channel for oxidant delivery.

Contemporaneous with our studies on the *E. coli* system, *C. ammoniagenes* NrdF containing a Mn^{III}_2 - Y^* cofactor was finally isolated from its endogenous host in sufficient quantities for its metallocofactor to be characterized.^{13,159} Detailed EPR analysis and a high-resolution crystal structure, combined with the results from the *E. coli in vitro* assembly system, supported the presence of a Mn^{III}_2 - Y^* cofactor in both *C. ammoniagenes* and *E. coli* NrdFs. Shortly thereafter, *E. coli* NrdF was isolated from *E. coli* grown under Fe-limited¹⁴¹ and oxidative stress⁶⁶ conditions, and *B. subtilis* NrdF was purified from its native organism.¹⁴² Both were shown to contain Mn^{III}_2 - Y^* cofactors. Thus the physiological relevance of the Mn^{III}_2 - Y^* cofactor is now clear, consistent with the early studies indicating dependence on Mn for corynebacterial growth.

Cofactor self-assembly: are additional factors needed?

Subsequently, *in vitro* assembly of Mn^{III}_2 - Y^* cofactor in NrdF from Mn^{II} , O_2 , and NrdI_{hq} and of diferric- Y^* cofactor from Fe^{II} and O_2 has been demonstrated for *B. subtilis*,¹⁴² *B.*

*anthracis*¹⁶⁰ and *B. cereus*.¹⁶¹ The ability to form active, dimanganese and di-iron cofactors is likely a general feature of the class Ib RNRs and may have interesting biological implications. In all cases reported thus far, the activity of the $\text{Mn}^{\text{III}}_2\text{-Y}^*$ NrdF is 5–10 times higher than that of $\text{Fe}^{\text{III}}_2\text{-Y}^*$ NrdF. However, in *in vitro* studies to date, only 0.25–0.6 Y^*/β_2 are generated and 1.4–1.8 Mn/β_2 are oxidized, far less than the theoretical maximum of 2 Y^*/β_2 and 4 Mn/β_2 . It is possible that the sub-optimal $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor assembly results from use of NrdI stoichiometrically for *in vitro* cluster assembly rather than catalytically, as it is used inside the cell^{141,142} with a yet to be identified reductase. Alternatively, optimal metal loading may require additional protein factors. By contrast, diferric- Y^* assembly has been optimized over many decades of study at $\sim 1 \text{ Y}^*/\beta_2$ and $\sim 3.5 \text{ Fe}/\beta_2$ in class Ia RNRs. As with $\text{Mn}^{\text{III}}_2\text{-Y}^*$ NrdF, more metal is oxidized during cluster assembly than is associated with Y^* . Furthermore, whether 1 or 2 Y^*/β_2 represents the cofactor loading *in vivo* is still unclear; inherent instability of the Y^* makes it impossible to address this question directly by purification of β_2 subunits from endogenous levels. Clearly, unresolved issues remain even after four decades of study of the assembly of the diferric- Y^* cofactor.

Recent work has hinted at a possible solution to these issues. In the case of the class Ib RNR, the presence of *nrdI* in an operon with *nrdEF* ultimately led to its identification as an essential accessory factor in $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor assembly. Similarly, the *E. coli* class Ia RNR *nrdAB* genes are present in an operon with a gene, *yfaE*, which is co-transcribed with *nrdB*¹⁶² and was predicted to encode a putative ferredoxin. Studies from our lab have recently shown that YfaE is a [2Fe2S]-ferredoxin-like protein that can, in fact, transfer electrons to NrdB,¹³² suggesting that the protein could be involved in provision of the extra electron required in cofactor assembly (Fig. 5A). In support of this hypothesis, cluster assembly *in vitro* with reduced YfaE in excess led to 1.5 Y^*/β_2 , and the stoichiometry of assembly suggested the extra reducing equivalent for assembly derived from YfaE.¹³² Furthermore, when cells overexpressing NrdB that was fully iron loaded were titrated with reduced YfaE, $\sim 2 \text{ Y}^*/\beta_2$ were formed, the highest levels of Y^* ever observed.¹⁶³ By contrast, Fe^{II} , ascorbate, and thiols act as kinetically efficient electron donors to NrdB during *in vitro* diferric- Y^* assembly and yet lead only to $\sim 1 \text{ Y}^*/\beta_2$. Therefore, while supporting YfaE's role in electron delivery, these experiments also suggest that YfaE has an additional function in cluster assembly, as reinforced by genetic experiments discussed below. These results from class Ia and Ib systems highlight a general caveat: even when a metal cofactor can self-assemble *in vitro*, there may be other protein factors involved in cofactor formation *in vivo* that must be identified. In the absence of these factors *in vitro*, self-assembly may be sub-optimal.

Control of metallation of class I RNRs

The fact that two different cofactors generated from different oxidants and requirements for reductant can be assembled from the same active site raises the issue of whether both, or only one, can be used in the wide range of environments an organism encounters. In the remainder of this review, we consider the implications of these observations for the physiological relevance of these two cofactors in the class Ib RNRs.

As noted above, how the class I RNRs successfully discriminate between Fe^{II} and Mn^{II} *in vivo* may be a consequence of relative affinities and metal availability. Unfortunately, metal availabilities are difficult to determine experimentally, as laboratory growth conditions rarely mimic physiological conditions and the speciation of “bioavailable” metals inside the cell is largely unknown. For pathogens, the situation is even more complex, as the host organism influences metal homeostasis for the pathogen,¹⁰⁴ making selection of an appropriate macrophage or animal model key to extraction of physiologically relevant information. However, unlike the enzymes discussed earlier in this review, class I RNRs have an additional filter for selectivity between Fe^{II} and Mn^{II} : the use of accessory factors

involved in conversion of the reduced, inactive form of the protein to the oxidized, active form. Work with class Ib RNRs described above has made it clear that NrdI plays an essential role in $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor assembly *via* electron donation to O_2 to produce the active oxidant. Studies of YfaE suggest the involvement of accessory factors in class Ia RNR cluster assembly *in vivo* as well. While there remain many issues to resolve, we suggest that aerobic prokaryotes have evolved three general strategies to ensure correct metallation of their class I RNRs depending on their general metal homeostatic machinery and RNR expression patterns. First, organisms containing both class Ia and Ib RNRs control RNR expression such that the class Ib is only expressed in Fe-limited or oxidative stress conditions. Second, organisms containing only class Ib RNRs tend to accumulate high levels of Mn^{II} , which may dictate the cofactor used by the RNR. Third, organisms containing class Ic RNRs, which are not present in the same organisms as class Ia or Ib RNRs, likely have more complex considerations if the $\text{Mn}^{\text{III}}\text{Fe}^{\text{IV}}$ cofactor is physiologically relevant.

Organisms containing both class Ia and Ib RNRs

Facultative aerobes that express both class Ia and Ib RNRs are almost exclusively enterobacteriaceae, like *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). Under normal laboratory growth conditions, the levels of intracellular Mn in *E. coli* have been measured to be $\sim 15 \mu\text{M}$,^{45,71} whereas iron levels are $\sim 0.1\text{--}1 \text{ mM}$.^{45,164} To consider whether differential bioavailability is a plausible model for RNR metallation in these systems without need to invoke specific Fe^{II} delivery factors, the K_{d} s of apo-NrdB for Fe^{II} and Mn^{II} and kinetics of metal binding and reaction with oxidant have to be considered. Unfortunately, this information is incomplete. The Hendrich lab has reported K_{d} s for Mn^{II} binding to *E. coli* apo-NrdB of 2 and $26 \mu\text{M}$ (Table 1), assigned to metal sites 2 and 1 (Fig. 1C), respectively.⁵⁸ Both K_{d} s are in the range of typical Mn concentrations in the cell in defined medium.⁷¹ The affinity of the protein for Fe^{II} is unknown, but, based on the qualitative observation that Mn^{II} inhibits diferric- Y^* assembly,¹⁵⁸ it is probably lower than for Mn^{II} . Without knowledge of the K_{d} s for Fe^{II} binding and of metal speciation in the cell, the state of class Ia RNR metallation under the physiological conditions of $\sim 0.1\text{--}1 \text{ mM}$ iron^{45,164} and $\sim 2 \mu\text{M}$ β_2 cannot be predicted.¹⁶³ It is possible that Fe^{II} binding could outcompete Mn^{II} without requiring a chaperone. In support of the idea that Fe^{II} can be readily pulled from the labile iron pool and cluster assembled, up to $\sim 120 \mu\text{M}$ β_2 is completely loaded with iron in rich media when NrdB is expressed from a titrable, arabinose-inducible promoter.¹⁶³ Furthermore, the reactivity of the Fe^{II}_2 cluster with O_2 relative to Mn^{II}_2 and mixed $\text{Mn}^{\text{II}}\text{Fe}^{\text{II}}$ clusters^{158,165} could allow sufficient time for inappropriately loaded metals to dissociate and re-metallate correctly. Our recent results suggest that even when *E. coli* are grown under severe Fe limitation with Mn^{II} supplementation, conditions under which NrdF is expressed and active,¹⁴¹ NrdB is also expressed. However, its activity is very low, likely due to Mn^{II} loading. When Fe^{II} levels are increased in the growth media, mismetallation of NrdB may be reversible.

The correct metallation of the *E. coli* class Ia RNR may also be aided by the accessory factor, YfaE (Fig. 7). In addition to the biochemical data presented above pointing to an important role for YfaE in diferric- Y^* cluster assembly, Martin and Imlay⁶⁶ have recently found that *yfaE* is essential for NrdB's discrimination between Fe^{II} and Mn^{II} during oxidative stress conditions ($0.5\text{--}1 \mu\text{M H}_2\text{O}_2$), but not under iron limitation or normal growth conditions. As with the biochemical data, a simple role in provision of the extra electron required in cofactor assembly (Fig. 5A) does not seem sufficient to explain this observation. One possibility is that YfaE could act as an iron chaperone to NrdB, as has been discussed elsewhere.^{30,166} A second possibility is that YfaE binding to NrdB could influence the kinetics of Fe^{II} and/or O_2 access; the rate-limiting step in formation of the tyrosine oxidizing intermediate **X** (Fig. 5A) in class Ia RNR cofactor assembly *in vitro* is a conformational

change associated with Fe^{II} binding.^{167–169} Further studies are required, however, to understand YfaE's role in *E. coli* NrdB assembly. Intriguingly, YfaE is not essential in most growth conditions, nor is it universally conserved in organisms containing class Ia RNRs (unlike NrdI for class Ib RNRs). This suggests that other organisms have evolved distinct, though possibly related, strategies to optimize class Ia cluster assembly *in vivo*.

In contrast to the class Ia RNR, under “normal” intracellular iron levels in *E. coli*, the class Ib RNR is not significantly expressed.^{170,171} Early studies^{171–173} showed that iron limitation and oxidative stress lead to upregulation of the class Ib operon (*nrdHIEF*; *nrdH* encodes a reductant for NrdE). Our purification of active NrdF from an iron-limited *E. coli* strain¹⁴¹ and genetic and whole-cell EPR studies of Martin and Imlay⁶⁶ of *E. coli* experiencing oxidative stress⁷¹ have together shown that the class Ib RNR is expressed, is active, and contains a Mn^{III}₂-Y* cofactor under these conditions. The mechanisms by which this occurs in both cases are linked, as shown in Fig. 3.

Iron limitation is sensed in *E. coli* by the Fe^{II}-responsive transcriptional regulator Fur, usually by binding of Fe^{II}-Fur to a sequence upstream of the promoter region to repress transcription of a wide range of genes involved in iron homeostasis. Fe^{II}-Fur also positively regulates certain genes, such as Fe-SOD, a process that is mediated posttranscriptionally by the small RNA RyhB, which is itself negatively regulated by Fe^{II}-Fur.¹⁷⁴ In low Fe^{II} conditions, when Fur is largely in the apo form, Fur's affinity for its binding site decreases, and genes in its regulon, such as the *nrdHIEF* operon^{66,175,176} and *mntH*,^{65,177} are transcribed. MntH expression subsequently leads to Mn^{II} import, ultimately facilitating loading of NrdF with Mn^{II}.

Oxidative stress also favors Mn^{III}₂-Y* cofactor formation in NrdF. One of the sensors of oxidative stress in *E. coli* is the transcriptional regulator OxyR. H₂O₂ stresses of >0.1–0.2 μM lead to oxidation of two Cys residues to a disulfide bond on OxyR,⁶⁷ activating transcription of various genes involved in oxidative stress response, including *dps* and *mntH* (Fig. 3). As described above, Imlay and coworkers have proposed that H₂O₂ also leads to oxidation of the mononuclear Fe^{II} cofactors of many enzymes such as Rpe to Fe^{III}, which dissociates, inactivating the enzymes.^{15,25} H₂O₂ also inactivates Fe^{II}-Fur *in vivo*, plausibly by a similar mechanism of oxidation of Fur-bound Fe^{II} (although no biochemical studies of this have been reported), thus mimicking iron limitation and leading to derepression of Fur-regulated genes.⁶⁴ To combat these effects, iron is sequestered by the ferritin-like protein Dps to limit the Fenton chemistry associated with iron and H₂O₂, and Mn^{II} import may serve to protect the normally Fe^{II}-enzymes from oxidative damage. Recent work has also indicated that *nrdHIEF* is regulated by apo-IscR.⁶⁶ IscR contains a [2Fe2S] cluster in its holo form and negatively regulates the “housekeeping” iron sulfur cluster biosynthesis pathway, Isc.¹⁷⁸ Oxidative stress disrupts this pathway, possibly initially by destruction of the [2Fe2S] cluster on IscR, leaving the apo form,⁶⁸ although again no biochemical studies of the effect of H₂O₂ on [2Fe2S]-IscR have been reported. Apo-IscR can act as a positive regulator of other genes, such as the backup Suf iron-sulfur cluster assembly system and *nrdHIEF*.¹⁷⁹ Therefore, under oxidative stress or Fe limitation, multiple pathways converge to create conditions favoring metallation of NrdF with Mn^{II}.

These results suggest that NrdF is only expressed when it can be correctly metallated with Mn. Therefore, correct metallation of *E. coli* NrdB and NrdF may be ensured by controlling the expression patterns of these proteins in response to metal availability. We suggest this will be true for other organisms that contain both class Ia and Ib RNRs as well. This hypothesis is supported by studies of class Ia and Ib RNR mutants of the related pathogen *S. Typhimurium* during infection of macrophages. Gibert and coworkers¹⁷⁶ found that NrdEF, but not the class Ia NrdAB, plays a key role in early stages of infection (up to 6 h).

However, a *S. Typhimurium* $\Delta nrdAB$ mutant is inviable at 24 h (once the pathogen's virulence response has been induced¹⁸⁰) unless the macrophage is lacking Nramp1, the divalent metal transporter responsible for efflux of metals from the phagosome, and the cells are exposed to an iron chelator. Although these experiments were conducted before the physiological relevance of the Mn cofactor of NrdF in *E. coli* was reported, one interpretation is that NrdEF can sustain growth only as long as Fe levels are low, either because NrdF is not expressed at high enough levels in Fe-sufficient conditions,^{171,181} or because the activity of $\text{Fe}^{\text{III}}_2\text{-Y}^*$ NrdF, if it can form *in vivo*, is too low.^{12,66} These data suggest that NrdF is activated only by manganese *in vivo* in these organisms.

Organisms containing only class Ib RNRs

Unlike *E. coli*, many of the numerous prokaryotes that encode class Ib RNRs as their sole class I RNRs contain high concentrations of manganese even under normal growth conditions, and we suggest that this may drive Mn loading of their class Ib RNRs. *Lactobacillus plantarum*,⁵² *Deinococcus radiodurans*,^{182,183} *B. subtilis*,⁶² and *Staphylococcus aureus*,¹⁸⁴ accumulate 20 mM, ~200 μM , ~100 μM , and >50 μM Mn^{II} , respectively, under “normal” laboratory growth conditions.¹⁸⁵ In these organisms, the presence of high levels of Mn^{II} would likely readily outcompete Fe^{II} loading of a class Ia RNR, which may be reflected in the utilization of a class Ib RNR. In fact, *C. ammoniagenes* restricts incorporation of Fe into NrdF when the protein is overexpressed in its native organism in medium containing 185 μM Fe with no added Mn.^{13,159} The *B. subtilis* class Ib RNR also contains a $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor even when grown in rich medium, albeit when it is overexpressed 35-fold.¹⁴²

It is also possible that metallation of certain class Ib RNRs may be analogous to non-redox Fe/Mn enzymes in that NrdF could be loaded and active with both dimanganese and diiron cofactors in different growth conditions in the same organism. One would expect this situation to be most likely in organisms whose NrdFs are comparably active with $\text{Mn}^{\text{III}}_2\text{-Y}^*$ and $\text{Fe}^{\text{III}}_2\text{-Y}^*$ cofactors. The first such organism is *Streptococcus sanguinis*.¹⁸⁶ Although available intracellular Fe and Mn concentration data for this and other streptococci are not easily translatable to concentrations (μM) for comparison with the metal concentrations mentioned above, the related *S. pneumoniae* has been reported to accumulate similar levels of Mn and Fe (300 ng mg^{-1} protein),¹⁸⁷ suggesting the plausibility of metal loading and assembly of both Mn and Fe cofactors *in vivo*. Other studies of *S. pneumoniae* have reported somewhat higher Fe concentrations (900 ng mg^{-1} protein).¹⁶⁴ However, the speciation of these metals (*i.e.*, whether the two metals are equally “bioavailable”) is unknown. If both metals are similarly bioavailable in certain conditions, the organism may tolerate loading of a fraction of its class Ib RNR with iron, as occurs with partial mismetallation of MnSOD with Fe even when the Fe-loaded MnSOD is inactive.^{21,22}

The dependence on NrdI for $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor assembly provides a unique handle by which the question of metal loading of NrdF *in vivo* can be assessed without requiring purification of active NrdF from many growth conditions. Our favored interpretation of the universal conservation of *nrdI* is that the $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor is relevant in all organisms in at least some set(s) of growth conditions. However, because NrdI can also transfer electrons to $\text{Fe}^{\text{III}}_2\text{-NrdF}$ *in vitro*,¹⁴⁰ it is also possible that NrdI acts as the extra electron source for $\text{Fe}^{\text{III}}_2\text{-Y}^*$ cofactor assembly in some organisms under certain growth conditions (Fig. 7). As the $\text{Fe}^{\text{III}}_2\text{-Y}^*$ cofactor can self-assemble *in vitro* from Fe^{II} and O_2 alone, it is expected that assembly of this cluster could still proceed *in vivo* in the absence of NrdI using cellular thiols, ascorbate, or generic flavodoxins or ferredoxins¹⁸⁸ as a donor of the “extra” electron in the assembly process. In contrast, deletion of *nrdI* would be expected to be lethal for organisms whose NrdF uses Mn only.

Only three studies of the essentiality of NrdI have been carried out to date. Gene inactivation studies have indicated that *nrdI*, for example, is essential for growth of *B. subtilis*¹⁸⁹ and *S. sanguinis*¹⁹⁰ in rich media. More detailed studies have been recently reported by Sjöberg and coworkers on the *nrdI* genes of *Streptococcus pyogenes*, which utilizes only class Ib RNR genes for aerobic growth.¹³⁹ Their reported experiments are complicated by two factors. First, *S. pyogenes* contains two sets of class Ib RNR genes, only one of which is active *in vitro*. Second, a heterologous complementation assay in rich media in an *E. coli* strain unable to reduce nucleotides aerobically was used to assess the activity of the *S. pyogenes* genes *in vivo*. While they concluded from their studies that one of the *nrdI* genes, denoted *nrdI**, is essential, the experimental results are sufficiently complex to remain inconclusive. However, use of standard genetic and biological methods including deletion strain construction, homologous complementation assays, and *in vivo* assays of virulence for the many pathogens that depend on class Ib RNRs should enable assessment of the essentiality of NrdI in physiological growth conditions in many organisms. We anticipate that it will be found to be, suggesting that class Ib RNRs are manganese enzymes in general.

Metallation of class Ic RNRs

Metallation of the *C. trachomatis* class Ic RNR must be more complex than for class Ia and Ib RNRs because of its heterodinuclear cofactor and the reactivity of the Fe^{II}_2 form with O_2 to generate a likely inactive $\text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}$ cofactor. *In vitro* reconstitution studies from apo-NrdB^{23,144,191} or overexpression of NrdB^{192,193} in *E. coli* grown in rich medium supplemented with Mn^{II} have demonstrated challenges associated with loading two metals. Both studies have resulted in crystallographic characterization of NrdB with distinct results. In the NrdB reconstituted with Mn and Fe *in vitro*, Mn appears to be present at both sites 1 and 2 (although mainly at site 1),¹⁹¹ whereas in the NrdB expressed recombinantly in the presence of added Mn^{II} , Mn appears to be localized to site 1.¹⁹³ However, the former results indicate that the method of reconstitution impacts the metal distribution in the oxidized protein, whereas the interpretation of the latter results is complicated by the presence of Mn^{II} and Pb^{II} in the crystallization conditions. *C. trachomatis* is an obligate intracellular parasite and isolation of its RNR from its endogenous environment in sufficient amounts to characterize the cofactor biosynthesized *in vivo* likely presents an insurmountable challenge. Thus identification of a class Ic RNR from a more tractable organism whose physiological niche is understood may allow biochemical and biological studies of this fascinating metallation problem.

Synthesis and conclusions

The examples presented in this brief review illustrate the diversity of Fe/Mn interchange reactions in biology. In each case, the specific role of the metal ion in catalysis dictates the specific strategy used for the switch. For non-redox Lewis acid enzymes that use Fe^{II} or Mn^{II} as a cofactor, the metal can be simply exchanged. For dioxygenases, if in fact they are redox active, use of an electron-rich substrate may allow Mn^{II} to be used in place of Fe^{II} . For SODs, a similar but distinct active site is necessary to perturb the redox potentials to bring them in the range for catalyzing both reduction and oxidation of $\text{O}_2^{\bullet-}$. For RNRs, a different oxidant is used to overcome the inability of O_2 to oxidize Mn^{II} ; the same protein can be used, but only if the unique accessory protein NrdI is present to produce that oxidant. Beyond demonstrating Nature's remarkable chemical creativity, we have discussed these systems together to illustrate that, in the absence of functional reasons to use one specific metal ion over another, an organism's utilization of Mn over Fe or *vice versa* for a particular enzyme is likely a response to environmental factors that will also be reflected in the metallation of other enzymes that can use either Mn or Fe. In this view, in *E. coli*, replacement of Fe^{II} with Mn^{II} in Rpe in oxidative stress conditions,²⁵ upregulation of MnSOD in Fe limitation,⁹⁷ and expression of the Mn-dependent class Ib RNR in the same

conditions^{66,141} are not isolated phenomena but reflections of a shift to a more Mn dependent metabolism in these conditions (Fig. 3). Many of the Fe-dependent extradiol dioxygenases purified to date are from pseudomonads, which contain Fe-dependent class Ia RNRs. However, the few dioxygenases likely to be Mn-dependent *in vivo* are from organisms like *Arthrobacter*, whose class Ib RNR is also likely to be Mn-dependent,¹⁹⁴ or *Brevibacillus brevis*, whose class Ib RNR is closely related to that of *B. subtilis* and therefore may also be manganese dependent. These observations may not be coincidental. We suggest that the metal usage of these diverse protein systems is linked, reflecting the overall metal usage by the organism. Further work is necessary to determine if these proposed connections are valid and generalizable.

A number of issues have to be considered to propose a global model for how Nature manages metal homeostasis to minimize mismetallation, despite the use of protein scaffolds that exhibit little inherent metal ion specificity. Ultimately, the answers to this question will require more complete information: total and bioavailable metal concentrations in a variety of organisms in different growth conditions, K_d s of the distinct metal ions for their respective proteins, cellular concentrations of these proteins, protein expression patterns, kinetics of cofactor assembly, speciation of “free” metal ions in cells, and whether metal chaperones for weakly binding metals like Mn^{II} and Fe^{II} exist in general. This is an ambitious undertaking but, as more organisms and protein systems are studied and new metallomic methods^{3,22,195,196} are applied, the issues of the cellular interplay between manganese and iron specifically and of mismetallation in general will come into focus.

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Biographies



Joseph A. Cotruvo, Jr

Joseph Cotruvo received his PhD in Biological Chemistry under the guidance of JoAnne Stubbe at MIT, where he was a National Defense Science and Engineering Graduate fellow. His graduate work focused on the mechanism of assembly of the dimanganesetyrosyl radical cofactor of class Ib ribonucleotide reductase, in vitro and in vivo. In the fall, he will continue pursuing his interest in the roles of metal ions in biological systems as a postdoctoral associate in the laboratory of Prof. Christopher Chang at the University of California, Berkeley. Joey's dog growing up was named after S-adenosylmethionine, before he knew it existed.



JoAnne Stubbe

JoAnne Stubbe is Novartis Professor of Chemistry and Biology at the Massachusetts Institute of Technology. Over the course of her academic career, her research interests have ranged from the bio-synthesis of purine nucleotides and of polyhydroxyalkanoates, to the mechanism of action of the anticancer drug bleomycin, to the mechanism of ribonucleotide reduction. She has been honored recently for her contributions with the National Medal of Science, the Welch Award in Chemistry, and the Franklin Medal. She is pictured with her dog, McEnzyme, named, of course, after one of the lesser known characters of "the Scottish play."

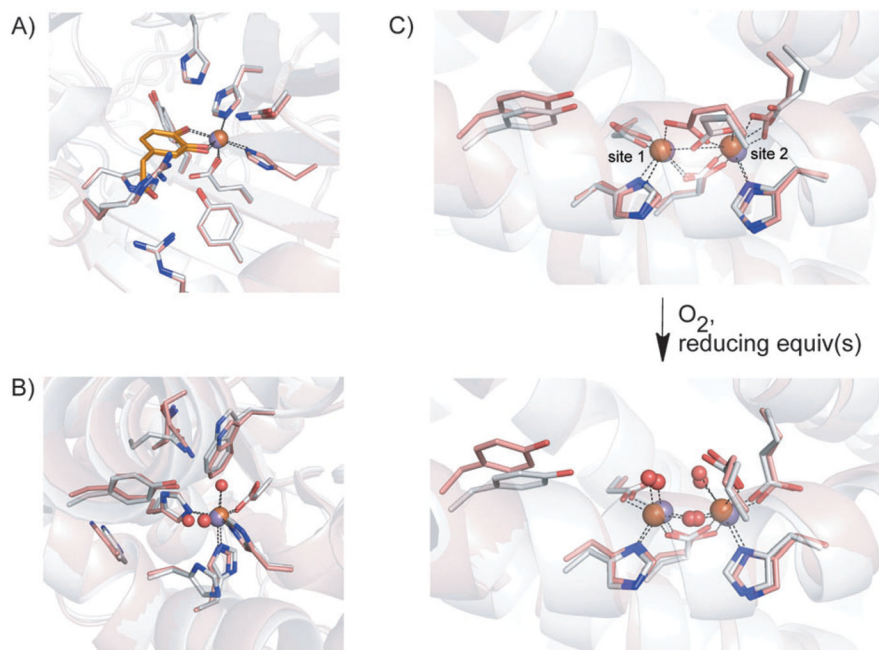


Fig. 1. Comparison of the metal binding sites of Fe- and Mn-containing extradiol dioxygenases (A), SODs (B), and the class Ia and Ib RNRs (C), which are the primary subjects of this review. (A) Active sites of the Fe-containing homoprotocatechuate 2,3-dioxygenase (HPCD) of *Brevibacterium fuscum* (white) (PDB code: 1Q0C) and the Mn-containing HPCD (MndD) of *Arthrobacter globiformis* (pink, 1F1V), each soaked with homoprotocatechuate (orange).³¹ (B) Active sites of the FeSOD (white, 1ISB)³² and MnSOD (pink, 1D5N)³³ of *E. coli*. (C) Reduced (top) and oxidized (bottom) forms of the class Ia and Ib RNRs. At the top, the diferrous form of *E. coli* class Ia RNR (1PIY, white)³⁴ is overlaid with the dimanganese(II) form of *B. subtilis* class Ib RNR (4DR0, chain A, pink).³⁵ At the bottom, the diferric form of *E. coli* class Ia RNR (1MXR, white)³⁶ is overlaid with the dimanganese(III) form of *C. ammoniagenes* class Ib RNR (3MJO, pink).¹³ Mn ions are purple spheres, Fe ions are brown spheres, and solvent molecules are red spheres. Metal-ligand bonds are shown as dotted lines. Figures were generated using PyMOL.

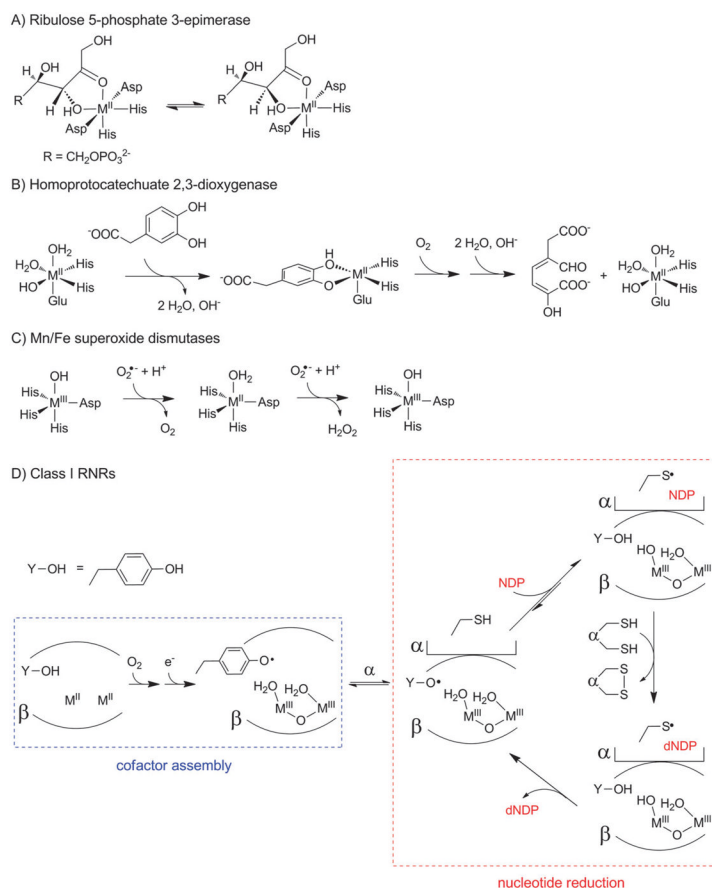


Fig. 2.

Reactions catalyzed by the enzymes discussed in this review, where M=Mn or Fe. (A) Ribulose 5-phosphate 3-epimerase (Rpe) catalyzes the reversible interconversion of ribulose 5-phosphate and xylulose 5-phosphate. (B) A representative of the extradiol dioxygenases, homoprotocatechuate 2,3-dioxygenases catalyze the oxidation of homo-protocatechuate to 2-hydroxy-5-carboxymethylmuconate semialdehyde. (C) Superoxide dismutases (SODs) catalyze the disproportionation of O₂^{•-} to O₂ and H₂O₂. (D) Ribonucleotide reductases (RNRs) catalyze the conversion of nucleoside 5'-diphosphates (NDPs) to deoxynucleoside 5'-diphosphates (dNDPs). The class I RNRs use two dimeric subunits, α₂ and β₂, in a 1 : 1 complex; only one monomer is shown here. The β₂ subunit contains the metal cofactor and the α₂ subunit contains the site of nucleotide reduction. Upon binding of substrate (NDP, shown) and nucleotide effector (not shown) to α₂, Y[•] oxidizes a Cys residue in the α₂ active site to a thiyl radical (S[•]), which initiates nucleotide reduction. Here we show the reaction of a class Ia RNR (M=Fe). The reactions of the class Ib and Ic RNRs are similar, with differences related to their distinct cofactors. In the class Ib RNRs (M = Mn), either O₂^{•-} or HOO(H) (not yet determined) is the oxidant in cofactor assembly. In the class Ic RNRs, the tyrosine shown for the class Ia and Ib RNRs is replaced by a redox-inert residue (Phe in *C. trachomatis* RNR) and the active enzyme employs a Mn^{IV}Fe^{III} cofactor in the β subunit.

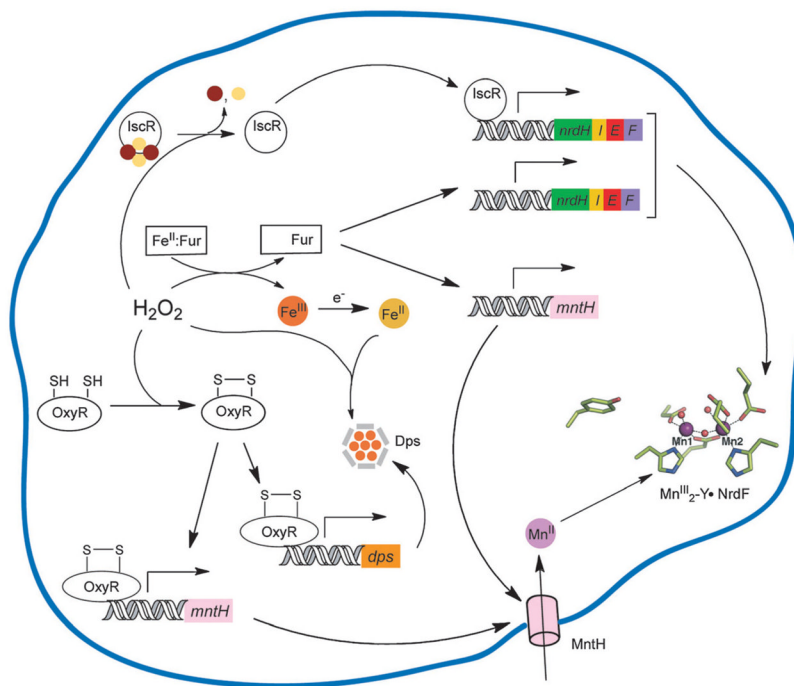


Fig. 3. Model for the interrelation of oxidative stress and Fe limitation in expression of the class Ib RNR in *E. coli*. In this proposal, H₂O₂ can inactivate Lewis acid-requiring enzymes that utilize Fe^{II} in catalysis by oxidizing Fe^{II} to Fe^{III}, which irreversibly dissociates (as a result of ligand modification, for example).^{15,25} Fur is also inactivated, presumably by a similar mechanism.⁶⁴ Loss of Fe from Fur activates transcription of Fur-repressed genes such as *mntH* and *nrdHIEF*, mimicking general Fe limitation.^{65,66} H₂O₂ also induces, *via* OxyR,⁶⁷ expression of MntH and the ferritin-like protein Dps. Dps sequesters free iron using H₂O₂ as an oxidant. MntH imports Mn^{II}. H₂O₂ leads to increased levels of the apo form of IscR, by destroying the [2Fe2S] cluster of IscR (shown here) and/or by interfering with its assembly (as proposed by Imlay⁶⁸); apo-IscR positively regulates *nrdHIEF* transcription.⁶⁶ Together, Mn^{II} import and Fe^{II} oxidation and sequestration leads to NrdF being metallated with Mn^{II}, with the essential oxidant for cluster assembly provided by NrdI's reaction with O₂.¹²

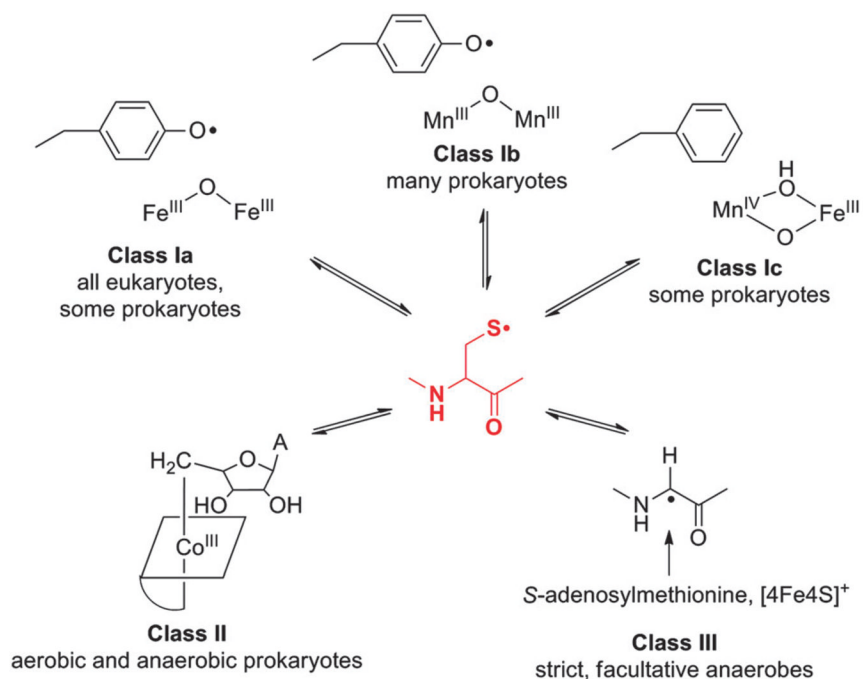
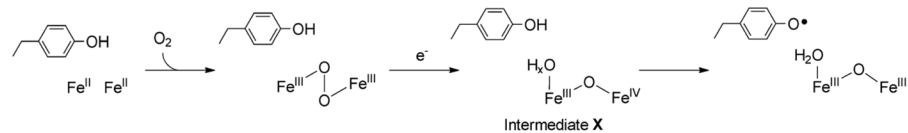
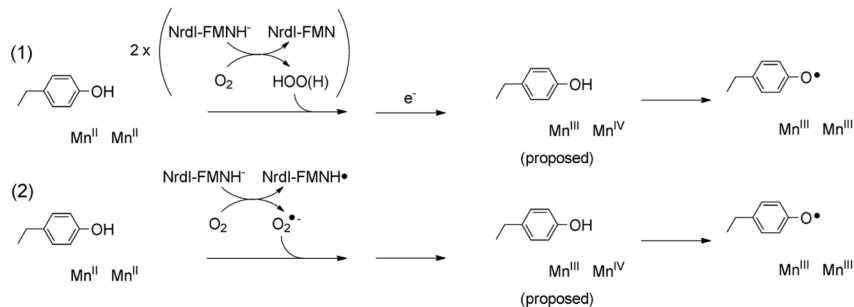


Fig. 4. Classes of RNRs. RNRs are classified on the basis of the metal cofactor used to reversibly generate the cysteine thiol radical (red) essential for catalysis. Class Ia RNRs use a diferric- Y^{\bullet} cofactor, class Ib RNRs use a dimanganese(^{III})- Y^{\bullet} cofactor, class Ic RNRs use a $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor, class II RNRs use adenosylcobalamin, and class III RNRs use a glycol radical generated by an activating enzyme requiring S-adenosylmethionine and a $[\text{4Fe4S}]^+$ cluster.

A) Diferric-tyrosyl radical cofactor assembly, class Ia (and Ib?)**B) Dimanganese(III)-tyrosyl radical cofactor assembly, class Ib****C) Manganese(IV) iron(III) cofactor assembly, class Ic****Fig. 5.**

General mechanisms of assembly of the metallocofactors of the class I RNRs. Two proposed mechanisms for $\text{Mn}^{\text{III}}_2\text{-Y}^\bullet$ cofactor assembly in class Ib RNRs are shown.^{12,13,30} The identities of the bridging ligands are only definitively established for the class Ia RNRs.

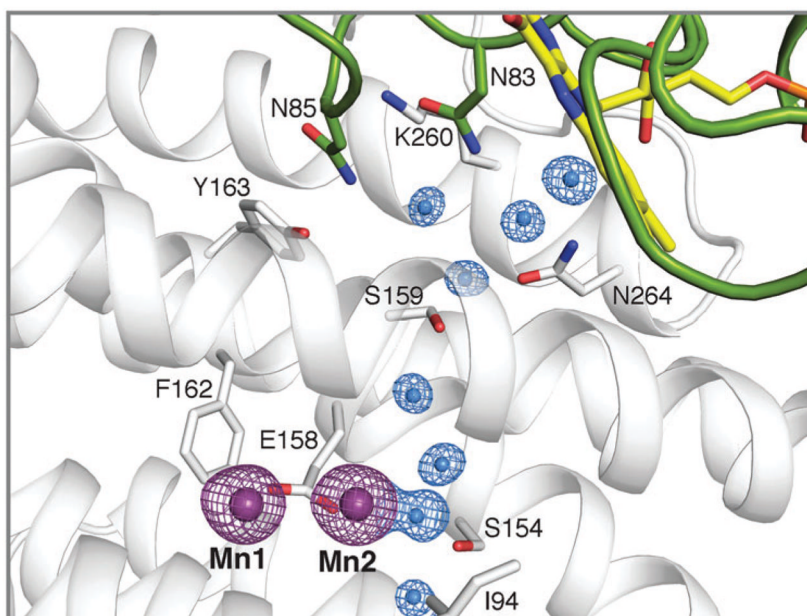


Fig. 6. The proposed oxidant access route for $\text{Mn}^{\text{III}}_2\text{-Y}^*$ cofactor assembly in the *E. coli* class Ib RNR complex of NrdI (green) and NrdF (gray) (PDB code: 3N3A). Water molecules in the channel connecting the FMN cofactor (yellow) of NrdI and the Mn^{II}_2 site (purple spheres) of NrdF are shown in blue spheres and mesh, and the highly conserved hydrophilic residues from NrdI and NrdF lining the channel are indicated.

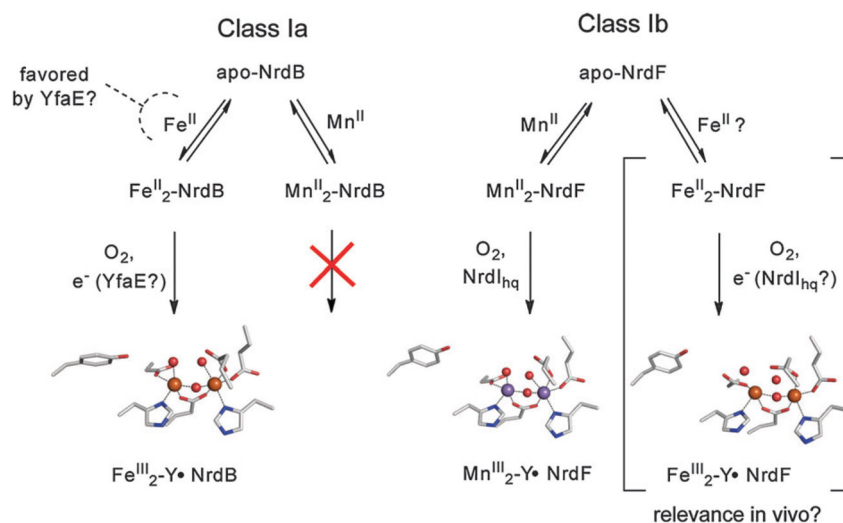


Fig. 7. The importance of accessory factors in metallation and assembly of the *E. coli* class Ia and Ib RNR metallocofactors. The structural images were created in PyMOL from the crystal structures of *E. coli* Fe^{III}₂-NrdB (1MXR), *C. ammoniagenes* Mn^{III}₂-NrdF (3MJO), and *S. Typhimurium* Fe^{III}₂-NrdF (2R2F).

Table 1

Dissociation constants of non-redox-active and redox-active enzymes and metal sensing transcription factors for Fe^{II} and Mn^{II}

Enzyme	K_d (μ M)		Active with metal?		Ref.
	Fe ^{II}	Mn ^{II}	Fe ^{II}	Mn ^{II}	
Non-redox (Lewis acid) catalysis					
<i>S. Typhimurium</i> PPP-family protein phosphatase PppB	1300 ^a	1, 3 ^a	Yes ^a	Yes	56
Redox catalysis					
<i>Ralstonia eutropha</i> TfdA ^b	7.5	6.8	Yes	No	57
<i>E. coli</i> MnSOD ^c	0.025	0.0003–0.003	No	Yes	2
<i>E. coli</i> class Ia RNR (NrdB)	ND ^d	2, 26	Yes	No	58
Uncertain redox role					
<i>Acinetobacter johnsonii</i> DkeI ^e	5.2	5.5	No?	Yes	59
Metal sensing					
<i>E. coli</i> Fur	1.2	24	Yes	Yes	60
<i>Rhizobium leguminosarum</i> Mur ^f	6 ^g	4 ^g	Yes	Yes	61
<i>B. subtilis</i> MntR	ND	160	ND	Yes	62
<i>B. anthracis</i> MntR	ND	60	ND	Yes	63

^aThese appear to be K_M values. Activity of the Fe enzyme is <5% of the Mn-loaded enzyme under the reported conditions. This enzyme is proposed to use Mn^{II} *in vivo* based on its significantly higher affinity for that metal.

^b2,4-Dichlorophenoxyacetic acid α -ketoglutarate dioxygenase.

^cMetal binding to SODs is generally irreversible at physiological temperatures, indicating a role for kinetics in addition to thermodynamics in metal loading of these enzymes.

^dND: not determined.

^e β -Diketone-cleaving enzyme, a cupin-type dioxygenase, carrying out a similar reaction to the extradiol dioxygenases discussed in this review.

^fManganese uptake regulator, related to Fur.

^gThe similarity of these K_d s raises the possibility that mismetallation of some transcription factors, like some enzymes, may be tolerated *in vivo*. Whether this occurs is unknown.

Table 2

Key moments in the determination of the identity of the cofactor of the class Ib RNRs. Unless otherwise noted, NrdF was purified from its endogenous organism

Year	Group	Organism	Mn/ β 2	Fe/ β 2	Y/ β 2	Specific activity ^d (nmol min ⁻¹ mg ⁻¹)	Ref.
1988	Auling, Follmann	<i>C. ammoniagenes</i>	NA	NA	ND	0.7	136
1994	Reichard	<i>S. Typhimurium</i> (expressed in <i>E. coli</i>)	NA	3.6	1	830	152
1996	Auling	<i>C. ammoniagenes</i>	NA	NA	0.13 ^b	0.9	153
1998	Sjöberg	<i>C. ammoniagenes</i>	0.9	0.16	ND	34	154
2000	Sjöberg	<i>S. Typhimurium</i> (reconstituted with Mn ^{II} and O ₂ or H ₂ O ₂)	3.2	NA	<0.02	3	117
		<i>S. Typhimurium</i> (reconstituted with Fe ^{II} and O ₂)	NA	3.2	0.4	325	117
2009	Auling	<i>C. glutamicum</i>	1.6	0.12	NA	32 000 ^d	155
2010	Stubbe	<i>E. coli</i> (reconstituted with Mn ^{II} , NrdI, O ₂)	1.4	0.03	0.25	600	12
		<i>E. coli</i> (reconstituted with Fe ^{II} and O ₂)	0.01	4–5 ^c	0.7	300	12
2010	Auling	<i>C. ammoniagenes</i>	1.5	0.06	0.35	69 000 ^d	13
2011	Stubbe	<i>E. coli</i>	0.9	NA ^e	0.2	720	141
		<i>B. subtilis</i>	1.8–2.4	0.14	0.4–0.5	52	142

ND: undetectable. NA: not determined.

^a All activity determinations used dATP as allosteric effector.

^b EPR signal observed, but differs substantially to those observed in ref. 12, 13, and 141, suggesting that this radical was likely not associated with a Mn^{III} 2-Y[•] cofactor.

^c Excess iron not removed following cluster assembly.

^d The reported activities of these proteins are 5–10-fold higher (up to 30-fold higher on a per-Y[•] basis) than for any other RNR purified to date, a result that requires verification.

^e EPR and UV-vis spectroscopic evidence suggested no significant amount of Y[•] was associated with iron.