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Class I Ribonucleotide Reductases: Metallocofactor Assembly and Repair In Vitro and In Vivo

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Abstract

Incorporation of metallocofactors essential for the activity of many enzymes is a major mechanism of posttranslational modification. The cellular machinery required for these processes in the case of mono- and dinuclear nonheme iron and manganese cofactors has remained largely elusive. In addition, many metallocofactors can be converted to inactive forms, and pathways for their repair have recently come to light. The class I ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides and require dinuclear metal clusters for activity: an Fe^{III}Fe^{III}-tyrosyl radical (Y•) cofactor (class Ia), a Mn^{III}Mn^{III}-Y• cofactor (class Ib), and a Mn^{IV}Fe^{III} cofactor (class Ic). The class Ia, Ib, and Ic RNRs are structurally homologous and contain almost identical metal coordination sites. Recent progress in our understanding of the mechanisms by which the cofactor of each of these RNRs is generated in vitro and in vivo and by which the damaged cofactors are repaired is providing insight into how nature prevents mismetallation and orchestrates active cluster formation in high yields.

Keywords

accessory factors/proteins; cofactor maturation; iron and manganese homeostasis; metal specificity; posttranslational modification; protein radicals

INTRODUCTION

New analytical methods are focused on identifying and quantifying modifications of proteins incorporated subsequent to their translation. One very important set of modifications, often overlooked, is the assembly of metallocofactors essential for enzymatic

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Very recent work from the Imlay lab coupling genetic studies to whole-cell EPR analysis has demonstrated that manganese is required for function of the *E. coli* class Ib RNR in vivo (163). Their results complement our biochemical studies demonstrating that *E. coli* NrdF purified from its native expression levels in iron-limited growth conditions contains a Mn^{III}Mn^{III}-Y• cofactor (124).

activity. An estimated one-third of proteins require metals for their function. Given the availability of the reduced metals and redox equivalents, many metallocofactors can “self-assemble” in vitro into their active form with varying degrees of success. However, recent studies from many groups have established the importance or essentiality of biosynthetic pathways for cluster insertion (1, 2) and have suggested the importance of repair or maintenance pathways (3–5) for regenerating active cofactors from damaged metal clusters. Pioneering studies of the Dean group (2), followed by work from many labs, have contributed to our understanding of iron-sulfur (FeS) cluster biosynthesis and the complex machinery required (reviewed in Reference 6). Complex metallocofactors, such as those found in nitrogenases (7) and hydrogenases (8, 9), require a large number of enzymes and scaffolding proteins for assembly. However, even for relatively simple metallocofactors containing copper (1, 10), nickel (11), cobalt (12, 13), redox-inert zinc (14), and perhaps other metals, the general belief is that a protein factor or peptide delivers the metal, as concentrations of these uncomplexed metals inside the cell are vanishingly low (14).

On the basis of this work, a number of generalizations are emerging concerning nature’s design and implementation of metallocofactor biogenesis. In this review, we initially provide an overview of the guiding principles for cluster assembly/repair and the challenges associated with their study. We then use our recent work on the class Ia and Ib ribonucleotide reductases (RNRs) and that of the Bollinger/Krebs lab on the class Ic RNRs as a model for nonheme diiron, dimanganese, and manganese/iron cluster assembly. These proteins use different metallocofactors despite nearly identical primary coordination environments of the metals, emphasizing the importance of biosynthetic pathways in correct metal insertion. Following a presentation of the structures of the metal clusters of each RNR subclass, we focus on the mechanism of cluster assembly in vitro and in vivo of each of the specific class members.

BIOSYNTHESIS AND REPAIR OF METALLOCOFACTORS

A number of general principles and challenges have emerged from studying metallocofactor formation with many different metals and levels of complexity.

Principles

1. The cofactors of many metalloproteins are likely generated by defined biosynthetic pathways.
2. Often (in bacteria), the proteins involved in the biosynthetic pathway are associated with the operon of the metalloprotein of interest, and thus, factors can be identified by genomic sequence analyses/comparisons.
3. Metals are transferred in their reduced state to facilitate ligand exchange between protein factors.
4. Specific protein factors include a metal insertase or chaperone to deliver the metal, specific redox proteins such as flavodoxins or ferredoxins that control the oxidation state of the metal, and GTPases or ATPases involved in protein unfolding/refolding to allow metal entrance into deeply buried active sites.

5. There is often biological redundancy in pathway factors (e.g., multiple ferredoxins), making it challenging to identify phenotypes with a simple gene deletion experiment; multiple deletions often are required.
6. There is likely a hierarchy of metal delivery to proteins, and the regulation of this hierarchy is not understood.
7. Compartmentalization (e.g., periplasm versus cytosol in prokaryotes) and relative affinities of protein coordination environments for various metals in relation to the intracellular concentrations of those metals (15) likely contribute to prevention of mismetallation.
8. Many proteins are never isolated from their native source but instead from heterologous expression systems, often leading to insertion of incorrect metals. Because the “gold standard” of activity is unknown, low activity associated with incorrect clusters may go unrecognized.
9. Metal clusters can become damaged by oxidants such as NO and $O_2^{\bullet-}$, and specific pathways are implicated in their repair.
10. Finally, during changes of oxidation state, ligands to the metal (e.g., His, Asp, Glu, and waters) can reorganize readily; structural rearrangements of carboxylate ligands (“carboxylate shifts”) are often critical to the cluster assembly process (16), and protons are often required for metal oxidation.

Examples of each of these principles are provided in the context of the RNRs described in this review.

Experimental Challenges

Generalizations can also be made about the experimental problems encountered in examining by biophysical methods self-assembly as well as biosynthetic and repair pathways for clusters generated from redox-active transition metals. In this review, we focus on Fe^{II} and Mn^{II} . The most abundant metal cofactors use iron, found in hemes, FeS clusters, mono- and dinuclear nonheme iron clusters, and a variety of specialty cofactors, such as those of nitrogenases and hydrogenases. The chemical properties of iron make it difficult to study. (a) Iron is commonly found in metallocofactors as either Fe^{II} or Fe^{III} . Fe^{IV} is often encountered transiently in the chemistry of cluster assembly. (b) Fe^{II} is rapidly oxidized under physiological conditions, with ligand-dependent rates. The resulting oxidation product, Fe^{III} , has limited solubility in aqueous solution at pH 7, and ligands to Fe^{II} are more exchange labile than those to Fe^{III} . Thus, iron is transferred as Fe^{II} in biosynthetic pathways. Oxidation of Fe^{II} can also produce reactive oxygen species ($O_2^{\bullet-}$, H_2O_2 , and $OH^{\bullet}$) that are potentially deleterious to the cell, making sequestration of iron by trafficking and storage proteins important. (c) Fe^{II} is weakly bound to proteins, making its interaction with proteins difficult to study owing to rapid dissociation. Often proteins that require Fe^{II} for activity are isolated in the metal-free (apo) state. (d) Fe^{II} is invisible to many biophysical methods, complicating observation of ligand reorganization and oxidation state changes. (e) Fe^{II} is ubiquitous inside the cell, making it difficult in vivo to monitor the Fe^{II} specifically associated with a protein of interest. These features of Fe^{II} have made it challenging, even

with the best-characterized FeS cluster biosynthetic pathways, to observe intermediates in metal cluster formation.

In contrast to iron, manganese has been established as essential for relatively few redox enzymes, mostly in mononuclear [e.g., superoxide dismutase (SOD)] or dinuclear (e.g., manganese catalase, class Ib RNR) sites and in the Mn_4Ca cluster of the oxygen-evolving machine of photosystem II. Some properties of manganese relevant to this review are as follows: (a) Manganese is usually found in metallocofactors as Mn^{II} or Mn^{III} , but it also can transiently form Mn^{IV} during cluster assembly or catalysis (an exception is the stable $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor of the class Ic RNR). (b) Unlike Fe^{II} , Mn^{II} is not readily oxidized by O_2 . (c) Both Mn^{II} and Fe^{II} can function as Lewis acids in catalysis. Fe^{II} may well have played this role in the pre- O_2 world and may still serve this function in certain situations (17). With the appearance of O_2 and the redox reactivity of Fe^{II} , however, this role has become delegated to Mn^{II} or Mg^{II} . (d) Like Fe^{II} , the ligands of Mn^{II} are more exchange labile than those of Mn^{III} . However, Mn^{II} is a “harder” ion than Fe^{II} and has higher affinity for carboxylate-rich coordination environments, as in class I RNRs (18). (e) Mn^{II} and most dimanganese clusters (with the exception of some $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$) are amenable to analysis by electron paramagnetic resonance (EPR) methods. These themes are exemplified by the various class I RNR cofactors.

OVERVIEW OF RIBONUCLEOTIDE REDUCTASES AND THEIR METALLOCOFACTORS

RNRs catalyze the conversion of nucleoside 5'-di- or triphosphates (NDPs or NTPs) to deoxynucleoside 5'-di- or triphosphates (dNDPs or dNTPs) in all organisms (Scheme 1) and play a central role in nucleic acid metabolism (19). These enzymes are largely responsible for the regulation of the concentrations and relative ratios of the dNTPs, which govern the fidelity of DNA replication and repair. RNRs are regulated at many levels (recently reviewed in Reference 20). Some mechanisms are universal, whereas others are organism specific. All RNRs are allosterically regulated, with nucleotide binding sites controlling the specificity of substrate reduction (“S site”) and overall activity (“A site”) (21). The consequences of allostery are quaternary structural changes that modulate RNR activity (22–25). All RNRs are transcriptionally regulated as well; for example, NrdR is a global regulator of prokaryotic RNRs (26, 27). NrdR contains an ATP/dATP-binding domain, designated the “ATP cone” domain, which mimics the A site in the class Ia enzymes, suggesting that its regulation of RNRs may in part involve sensing of cellular ATP/dATP levels. A third mechanism of regulation of the class I RNRs likely involves control of the concentration of active metallocofactor through the biosynthetic and repair pathways (4, 5). Other organism-specific regulatory mechanisms that control the activity of RNRs include protein degradation, subunit compartmentalization, small unfolded proteins that function as inhibitors, and posttranslational modification by kinases (28).

RNRs are divided into three main classes on the basis of the metallocofactors required for nucleotide reduction. Many organisms have multiple RNRs, the expression of which is dependent on growth conditions. The class I RNRs share the same structural fold, utilize two types of subunits, designated α and β , and contain dinuclear metal clusters required for

catalysis (Figure 1). α houses the active site where nucleotide reduction occurs; β harbors the metallocofactor essential for initiation of nucleotide reduction. Class I is further divided into three subclasses based on the identity of the metal cluster. In class Ia, it is a diferric-tyrosyl radical ($\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$) (29); in class Ib, it is a dimanganese(III)- $\text{Y}\cdot$ ($\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$) (30–33); and in class Ic, it is proposed to be a $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor (34). Important features of each of these subclasses are summarized in Table 1. The quaternary structures of these proteins are actively being investigated (35) but are best understood in the case of the class Ia RNRs from *Escherichia coli* and mouse. There is a general consensus that in *E. coli* the active form is $\alpha_2\beta_2$. In eukaryotes, the quaternary structure is more complex and the active form has been proposed to be $\alpha_n(\beta_2)_m$ ($n = 2, 4, 6$ and $m = 1$ or 3) (22, 23). The class II and III enzymes also have structures similar to the class I α subunits but use different metallocofactors. The O_2 -independent class II RNRs use adenosylcobalamin and the anaerobic class III enzymes use a glycyl radical generated by a $[\text{4Fe4S}]^{1+/2+}$ cluster and S-adenosylmethionine (36).

All RNRs share a common catalytic mechanism in which the metallocofactor is either directly or indirectly involved in oxidation of a conserved cysteine in the active site of α to a thiyl radical ($\text{S}\cdot$) (37, 38). The $\text{S}\cdot$ then initiates a complex, radical-mediated reduction process (Figure 2) (36, 39). In class I (and II) RNRs, the two electrons required for substrate reduction are provided by two active site cysteines, which must be re-reduced after every turnover by an exogenous reducing system. Although the use of the $\text{S}\cdot$ for initiation of nucleotide reduction is conserved, the mechanism by which the $\text{S}\cdot$ is generated is not. In the class II and III RNRs, the cysteine is oxidized by direct hydrogen atom abstraction by a 5'-deoxyadenosyl radical or a glycyl radical, respectively. In the case of the class I RNRs, oxidation occurs by the $\text{Y}\cdot$ (class Ia, Ib) or $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cluster (class Ic) in the β_2 subunit over a long distance, proposed to be 35 Å, via a specific proton-coupled electron transfer (PCET) pathway involving conserved aromatic residues (Figure 3) (40, 41). The radical initiation process has been studied in the case of the class Ia (42–44) and Ic enzymes (45). In the former case, nucleotide reduction is rate limited by conformational changes triggered by the binding of substrates and effectors to α (46). The details of the mechanism of radical propagation between subunits are being unraveled by site-specific incorporation of unnatural amino acids into pathway residues (42, 43).

Among the class I RNRs, formation of the active metallocofactor has been best characterized in the class Ia enzymes. The general observations made with this cofactor class have recently been extended to the class Ib and Ic RNRs. First, self-assembly of class I RNR cofactors minimally requires apo- β_2 , Fe^{II} and/or Mn^{II} , oxidant, and a one-electron reductant, with class Ib also requiring an additional protein. It is our premise that the information learned from these studies is directly relevant to the requirements for cofactor biosynthesis. Second, the stability of the $\text{Y}\cdot$ s in class Ia RNRs is highly variable. The half-life of the $\text{Y}\cdot$ in *E. coli* RNR is four days, whereas that in human RNR is 25 min. Because the $\text{Y}\cdot$ is essential for catalysis, this instability has implications for the importance of repair (maintenance) pathways by which Tyr is reoxidized to the $\text{Y}\cdot$. As the half-life of the $\text{Y}\cdot$ of the human RNR is much shorter than the S phase of the cell cycle, for example, in which RNRs supply dNTPs for DNA replication, either the radical must be stabilized in vivo or

there must be a maintenance pathway to regenerate active cofactor. The remainder of this review summarizes our understanding of metallocofactor self-assembly and critically discusses our current knowledge of the biosynthetic pathways and the importance of the maintenance pathways in prototypes for each of the class I RNRs.

CLASS Ia RIBONUCLEOTIDE REDUCTASES

The formation of the $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor and its essential role in nucleotide reduction have been studied in detail in *E. coli*, *Saccharomyces cerevisiae*, and mouse. In *E. coli*, in contrast to most prokaryotes, the class Ia enzyme is responsible for supplying the deoxynucleotides required for DNA replication under normal aerobic growth conditions. The class Ia $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor is localized in β_2 (designated NrdB in *E. coli*) at the end of a hydrophobic channel proposed to be the access route for O_2 in cluster assembly (47–49). The structure of $\text{Y}\cdot$ -reduced (met) β_2 and its cofactor (47) are shown in Figures 1a and 4. In 1973, Atkin et al. (50) first demonstrated that incubation of apo- β_2 of *E. coli* with Fe^{II} , O_2 , and reductant resulted in self-assembly of the $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor, capable of supporting dNDP formation in the presence of α_2 (NrdA), substrate (NDP), and the appropriate effector. Enzyme activity was proportional to the concentration of the $\text{Y}\cdot$. The optimized in vitro assembly process yields 1.2 $\text{Y}\cdot$ and 3.3–3.6 Fe/β_2 ; the distribution of the $\text{Y}\cdot$ between the β monomers of the dimer has not been established. Mouse and human β_2 s can also self-assemble active cofactor with 1.2 (1.0) $\text{Y}\cdot$ and 3.2 (3.5) Fe/β_2 (51; Y. Aye & J. Stubbe, unpublished results). The small subunit of *S. cerevisiae* RNR is unusual in that it is a heterodimer of β and a modified β , designated β' , in which three of the ligands to Fe^{II} in β have been mutated (2 Tyr residues and an Arg in place of 2 His and a Glu) (see Figure 4) (52); consequently, β' is unable to bind Fe^{II} . Efforts to self-assemble an active cofactor in *S. cerevisiae* $\beta\beta'$ have been much less successful, with typical yields of 0.3 $\text{Y}\cdot$ and 1.3 $\text{Fe}/\beta\beta'$ (53).

Time-Resolved Biophysical Methods Have Led to a Model for Cluster Assembly In Vitro

Studies beginning in the early 1990s and continuing to the present, employing an array of biophysical methods [stopped-flow ultraviolet-visible (SF UV-vis) spectroscopy, rapid-freeze quench (RFQ) EPR, electron-nuclear double resonance (ENDOR), Mössbauer, and extended X-ray absorption fine structure (EXAFS) spectroscopies, reviewed in References 28 and 54], have led to a mechanism by which the $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ is assembled from Fe^{II} and O_2 (Figure 5a). The overall stoichiometry of this reaction is shown in Scheme 2. $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ assembly is best understood in *E. coli* β_2 (55–57), but studies on the mouse β_2 (58, 59) have led to a similar model, although the rate constants for the various steps are altered, allowing detection of additional intermediates. In vitro assembly requires Fe^{II} to access the buried metal binding site of apo- β_2 by an unknown route or routes, a process that is limited in both *E. coli* and mouse by protein conformational changes (60, 61). The details of the loading process also appear to be distinct. For example, Fe^{II} binding is cooperative in mouse (62) but noncooperative in *E. coli* NrdB, with Fe^{II} binding first to site 2 (63). Once loaded, diferrous ($\text{Fe}^{\text{II}}\text{Fe}^{\text{II}}$)- β_2 rapidly reacts with O_2 to generate a diferric peroxide (59, 60, 64). The diferric peroxide is then reduced by Trp48 (*E. coli* numbering, Figure 4a) to generate a Trp cation radical ($\text{Trp}^{+\cdot}$) and an $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ species, designated intermediate **X** (65, 66).

Trp48⁺ is rapidly reduced to Trp by an external reductant (Fe^{II}, ascorbate, thiols); this may occur via a Y• intermediate, perhaps at Tyr356, although this is not clear at present (67, 68). X oxidizes Tyr122 to Y• by a PCET process. Once the radical is produced, it is sufficiently stable to carry out many turnovers of NDPs to dNDPs, although, as noted above, the stability of the Y• is organism dependent. The molecular structures of the peroxide and X have been investigated by many spectroscopic methods and by many theorists, but both remain incompletely understood and a topic of active debate (64–66, 69). On the basis of these in vitro studies, a biosynthetic pathway in vivo would presumably require at least one protein or small molecule to deliver the Fe^{II} to apo-β2 and a protein to deliver the extra reducing equivalent required for the four-electron reduction of O₂ to H₂O (Scheme 2) (70). The two model organisms in which cluster biosynthesis has been investigated thus far are *S. cerevisiae* and *E. coli*. Both systems have been examined by genetic, biochemical, and biophysical approaches.

Biosynthetic Pathway in *Saccharomyces cerevisiae*

S. cerevisiae has served as an excellent model for understanding iron homeostasis because of the availability of gene knockouts in a common background, as well as the extensive whole-cell studies of subcellular localization of all proteins, mRNA levels, and protein-protein interactions. This organism has provided much insight into mechanisms of iron insertion into heme and FeS cluster assembly (6), with studies suggesting an essential and general regulatory role for FeS clusters in iron homeostasis (71). The mechanism of mono- and dinuclear non-heme iron cofactor biogenesis, however, has until recently been largely unexplored. Thus, *S. cerevisiae* initially seemed like an excellent system in which to investigate cluster assembly of eukaryotic RNRs in vivo. Our original working model was that β' was a chaperone protein that could deliver Fe^{II} to β via its carboxylate-rich C-terminal tail. This hypothesis was based on the current understanding of Cu^I delivery to copper-zinc SOD by the copper chaperone Ccs1. Ccs1 contains a domain homologous to SOD and a surface-exposed, flexible C-terminal tail implicated in Cu^I binding and transfer. The metal is delivered in the reduced state such that ligand exchange between the two proteins can occur rapidly (10). Unfortunately, the C-terminal tails of all βs, including β', are essential for interaction with α and consequently essential for NDP reduction, making functional disentanglement of in vivo roles in cluster assembly and nucleotide reduction challenging. As noted above, however, biochemical studies and in vivo analysis (72, 73) have subsequently established that ββ' is the active form of the subunit (53, 74), and although only β can assemble a Fe^{III}Fe^{III}-Y• cofactor, deletions in β' result in a significant growth impairment or lethality (53, 75, 76). In one β' deletion strain (53), the levels of β are elevated 15-fold versus wild type (wt) as determined by Western blot analysis, and although only a very small amount of Y• is generated (<0.005 Y•/β), this Y• is sufficient to account for the observed doubling time of the organism with dNTP production now rate limiting for its growth. These results leave open the possibility that β' is an iron chaperone to β that then becomes an integral part of the functional RNR.

Whole-cell EPR has been a valuable tool in studying Fe^{III}Fe^{III}-Y• biosynthesis in *S. cerevisiae*. The level of Y• is sufficiently high in wt strains that it is detectable by this technique at endogenous levels in various growth conditions (77). Quantitative Western blot

analysis of β and β' , quantitative whole-cell EPR analysis of the $Y^\bullet$, and cell counting together suggest that stoichiometric amounts of $Y^\bullet$ ($1/\beta\beta'$) are generated in vivo and that the concentration of $Y^\bullet$ is not modulated as a function of the cell cycle (73). Affinity-tagged versions of β integrated into the genome have allowed rapid (~ 4 h) purification of $\beta\beta'$ by affinity chromatography with $\sim 0.5 Y^\bullet/\beta\beta'$. Therefore, there must be some small molecule or protein factor that can rapidly reduce the $Y^\bullet$ in cell lysates. A similar observation has been made with *E. coli* lysates. An additional feature of *S. cerevisiae* is the ability to permeabilize cells, allowing for direct measurement of nucleotide reduction within whole cells (78). Permeabilized cells, complemented with genetic manipulations, may provide an excellent system to identify the factors associated with RNR cluster assembly, such as iron delivery agents.

Recent genetic and biochemical studies have provided insight into how iron imported into the cytoplasm in *S. cerevisiae* is delivered to iron-requiring proteins in general, possibly including RNR. Mitochondria play a central role in biosynthesis of all FeS clusters through the iron-sulfur cluster (ISC) pathway (6), with cytosolic iron-sulfur assembly (CIA) machinery also being important for proteins located in the cytoplasm and nucleus. A recent paper (79) links both pathways to nonheme diiron cluster assembly for the first time. Two monothiol glutaredoxins, Grx3 and Grx4, are suggested to use their unusual FeS cluster for a general role in intracellular iron trafficking. Grx3 and Grx4 form homodimers that contain a labile, subunit-bridging $[2Fe2S]$ cluster with two glutathione ligands (80). Because Grx3/4 is essential in the strain background studied, the experiments that infer the importance of these proteins in $\beta\beta'$ cluster assembly are dependent on conditional expression. Deletion of Grx3/4 impairs all iron-requiring reactions (including nucleotide reduction) in the cytosol, mitochondria, and nucleus despite high levels of cytosolic iron. Whether Grx3/4 is directly or indirectly involved in cluster assembly in $\beta\beta'$ is not yet understood, and identification of additional factors, such as Dre2 (81), and the detailed mechanism of cluster insertion are active areas of research.

Unstable FeS clusters are implicated in the delivery of iron and sulfur together by scaffolding proteins to FeS cluster-requiring metalloproteins (6). However, a function for a labile FeS cluster as a source of iron alone, presumably Fe^{II} , would be unprecedented. The cannibalization of an FeS cluster does have precedent in biotin synthase, the enzyme that inserts the sulfur into desthiobiotin to form biotin. It is proposed that biotin synthase's $[2Fe2S]$ cluster is destroyed on each turnover to deliver only sulfur (82, 83). The lability of the FeS clusters involved in metallocofactor formation in vivo has made their isolation and characterization challenging. This instability can result in Fe^{II} release, which can, in the case of diiron clusters (e.g., class Ia RNR), be used for self-assembly, albeit in an inefficient manner. Thus, the stoichiometry and kinetics of Fe^{II} transfer are important to investigate.

Biosynthetic Pathway in Prokaryotes

Are the mechanisms of $Fe^{III}Fe^{III}-Y^\bullet$ cofactor assembly in RNRs universally conserved? Information about factors involved in metallocofactor biosynthesis is often obtained from operon organization within genomes. One prokaryotic model organism is *E. coli*, for reasons similar to those outlined above for the choice of *S. cerevisiae* as a eukaryotic model.

Bioinformatic studies revealed the presence of a gene encoding a putative ferredoxin immediately downstream of *nrdB* (encoding the β subunit) in 29% of genomes analyzed (70). In *E. coli*, this protein is designated YfaE. This protein could be involved in the biosynthetic or maintenance pathways for cluster formation (Figure 5a).

Isolation of YfaE required its solubilization from inclusion bodies and refolding in the presence of a defined ratio of $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ and S^{2-} under anaerobic conditions. YfaE reconstituted in this way had 80% of its iron in a $[\text{2Fe2S}]^+$ cluster and the remaining 20% in a $[\text{4Fe4S}]^{2+}$ cluster, determined by Mössbauer spectroscopy (70). Kinetic studies in vitro have demonstrated that YfaE is kinetically competent to function in the maintenance pathway to reduce the diferric cluster to the diferrous form and to deliver the required reducing equivalent in the biosynthetic pathway (Figure 5a, Scheme 2). Once $\text{Fe}^{\text{II}}\text{Fe}^{\text{II}}\text{-NrdB}$ is generated, it is readily converted to active cofactor in the presence of O_2 and reductant. YfaE's ability to form a $[\text{4Fe4S}]^{2+}$ cluster (which contains 2 Fe^{II} per cluster), along with the recently discovered role of Grx3/4 in iron cluster assembly in yeast, suggested to us that YfaE might have an additional function, to deliver Fe^{II} to NrdB. Anaerobic incubation of reconstituted YfaE with stoichiometric apo-NrdB led to disappearance of half of the $[\text{4Fe4S}]^{2+}$ cluster and, following addition of O_2 , formation of $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor, as determined by Mössbauer and EPR spectroscopies (84). On the basis of these results, it was hypothesized that in vivo $[\text{4Fe4S}]^{2+}\text{-YfaE}$ might transfer two Fe^{II} to apo-NrdB, with the cluster decomposing to a $[\text{2Fe2S}]^{2+}$ cluster, which upon one-electron reduction could then provide the extra electron for cluster assembly.

Growth studies with a *yfaE E. coli* strain carried out in minimal media containing 10 mM hydroxyurea (HU), known to reduce the $\text{Y}\cdot$ to Tyr in vitro and in vivo, demonstrated that, although *yfaE* is not essential, the growth rate is reduced to one-third that of the isogenic wt strain (84). This result suggests that one of the two other ferredoxins or flavodoxins found in the *E. coli* genome can substitute for YfaE in its electron transfer role (85) and that Fe^{II} can be delivered by more than one mechanism. Thus, the general observation of functional redundancy in important processes in vivo appears to be relevant for the *yfaE* strain.

Monitoring Iron Movement In Vivo Using Whole-Cell Electron Paramagnetic Resonance and Mössbauer Spectroscopies

Whole-cell EPR studies of *E. coli* NrdB in 1977 revealed that only when RNR levels were elevated ~ 10-fold relative to normal levels when grown in rich medium could the $\text{Y}\cdot$ be observed (86). Because the $\text{Y}\cdot$ can be readily reduced in vitro and in vivo by HU, which is rapidly taken into the cell, the amount of $\text{Y}\cdot$ in whole cells can be easily quantitated after subtraction of the background EPR signal from HU-treated cells. In addition to having the unique organic radical that is an indicator of cofactor assembly, the class Ia RNRs also possess a diferric cluster whose Mössbauer parameters are distinct from those of the Fe^{II} and Fe^{III} species in the cellular iron pool and in other proteins. One can therefore monitor iron movement from the oxidized and reduced iron pools into the RNR cofactor by using Mössbauer spectroscopy. This method allows detection of all oxidation states of iron simultaneously and is sensitive to the electronic environments of the iron species present.

However, it is limited by its requirement for ^{57}Fe , necessitating that cells be labeled with that isotope, and for high concentrations of a given iron species (~ 0.5 mM) for detection.

Therefore, to monitor $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\bullet$ formation in *E. coli* by EPR and Mössbauer methods, overexpression of NrdB is required. *nrdB* was tagged at its N terminus, and the gene was placed in a plasmid under control of an arabinose-inducible promoter. Altering the concentrations of arabinose modulated the concentrations of NrdB from 2 μM to 1.7 mM (5). Quantitative determination of the $\text{Y}\bullet$ concentration by whole-cell EPR and the amount of NrdB per cell by western blot analysis under different growth conditions revealed sub-stoichiometric amounts of $\text{Y}\bullet$, 0.3–0.5 $\text{Y}\bullet/\beta 2$, even though the affinity-purified protein contained 3.4–3.7 $\text{Fe}/\beta 2$.¹ These results differ from the stoichiometric amounts observed in *S. cerevisiae* and suggest that the amount of $\text{Y}\bullet$ in *E. coli* might be modulated in vivo as a mechanism to regulate nucleotide reduction as a function of the cell cycle. Furthermore, the high iron loading suggests that the iron pool available for cluster assembly during growth is large and mobile. In support of this proposal, parallel whole-cell Mössbauer experiments revealed increased intensity of the signals associated with the diferric cluster of NrdB and decreases in both Fe^{II} and Fe^{III} pools subsequent to arabinose induction. The iron incorporated into NrdB in these conditions thus came from both ferrous and ferric sources.

E. coli contains four Fe^{II} and Fe^{III} transporters, four ferritins (iron storage proteins), and many siderophore transporters that could be involved in delivering iron to NrdB. In the case of ferritins or siderophores, the Fe^{III} would have to be reduced to Fe^{II} before transfer. In principle, deletion strains could be used to limit the mechanisms by which iron might be inserted into RNR under different growth conditions, allowing iron movement to be monitored by Mössbauer methods. In practice, there are two major problems. First, the levels of NrdB must be increased by several orders of magnitude for detection under normal growth conditions in rich medium. Second, restriction of iron uptake in these deletion strains has the potential to lead to iron limitation, which in *E. coli* induces expression of the class Ib RNR (87). Understanding regulation of expression levels of RNRs under different growth conditions is thus essential to the design of experiments to monitor iron movement. Studies to monitor NrdB cluster assembly in a variety of deletion strains have thus far been unsuccessful owing to biosynthetic redundancy and the requirement for NrdB overexpression, suggesting new methods and approaches may be needed to study this problem.

CLASS Ib RIBONUCLEOTIDE REDUCTASES

Class Ib RNRs possess metal-binding residues identical to those of class Ia RNRs and were long generally assumed to also contain an $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\bullet$ cofactor, despite indirect evidence that a manganese-containing cofactor might be used in vivo. However, recent work has directly demonstrated that these RNRs in fact utilize a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor inside the cell; the detailed mechanism of formation of this novel cofactor is yet to be worked out. The

¹Interestingly, titration of crude extracts of these cells with reduced YfaE results in generation of as much as 2 $\text{Y}\bullet/\beta 2$, whereas in vitro assembly methods give a maximum of ~ 1.2 $\text{Y}\bullet/\beta 2$, and whole cells give only 0.3–0.5 $\text{Y}\bullet/\beta 2$.

ability of these enzymes to also form active $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor in vitro raises the question of how correct metallation of the class Ib RNRs is ensured in vivo.

Distribution, Regulation, and Differentiation from the Class Ia Ribonucleotide Reductases

Class Ib RNRs are found in a wide range of facultative and obligate aerobic prokaryotes, including many human pathogens, such as *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Bacillus anthracis*, and *Staphylococcus aureus* (88). Although some prokaryotes depend on a class Ib RNR alone for aerobic growth, many others contain one or more RNRs in addition to the class Ib enzyme. Both class Ia and Ib RNRs are present in enterobacteria such as *E. coli* and *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium). In *E. coli*, the class Ib RNR is present at insufficient levels to support normal aerobic growth in the absence of the class Ia enzyme. The expression of the class Ib RNR is repressed by the transcriptional regulator Fur (89, 90), and its expression is induced by iron limitation and oxidative stress² (87, 92, 93), conditions commonly encountered by invading pathogens. It is also artificially induced by HU (92–94) and by deletion of the transcription factor NrdR (27). Thus, the class Ib RNR might play an important role in early stages of infection when a bacterium is engulfed by a macrophage. A recent study (90) has suggested that this may be true for *Salmonella* Typhimurium, although longer term (24 h) survival requires the class Ia RNR.

Like the class Ia RNRs, the class Ib enzymes are composed of two homodimeric subunits, $\alpha 2$ (NrdE) and $\beta 2$ (NrdF) (Table 1) (95, 96). Despite low sequence identity (~20% between *E. coli* NrdAB and NrdEF), the class Ia and class Ib RNRs are structurally homologous (Figure 1a). One major difference lies in the $\alpha 2$ subunit, which in the class Ib RNR lacks the N-terminal ATP cone domain(s) containing the activity site (A site) for allosteric regulation by dATP and ATP (95, 97, 98). A second distinguishing feature is the clustering of the *nrdE* (α) and *nrdF* (β) genes with two other genes, *nrdH* and *nrdI*. In many organisms, such as *E. coli*, the operon is organized as *nrdHIEF*, and the genes are cotranscribed (93, 94). In other organisms, one or more of these genes is located elsewhere in the genome. In *Corynebacterium ammoniagenes*, for example, *nrdF* is located 1 kb downstream of *nrdHIE*, and the two regions are transcribed separately from their own promoters, but in a coordinated fashion (99). Other organisms, such as mycobacteriaceae and streptococci, contain two homologous copies of one or more of *nrdH*, *nrdI*, *nrdE*, and *nrdF*, but some of the translated proteins are nonfunctional in nucleotide reduction in vitro or in vivo (100, 101).

Until recently, the functions of NrdH and NrdI remained uncharacterized. NrdH is a small, 9-kDa protein containing a CXXC motif (usually C-[V/M]-QC) characteristic of thioldisulfide oxidoreductases. It has a glutaredoxin-like sequence but is thioredoxin-like in structure (102). It is efficiently reduced by thioredoxin reductase but not glutaredoxin reductase (103). Biochemical studies have demonstrated that NrdH can act as an electron donor to NrdE (103, 104), suggesting that it plays a role analogous to that of thioredoxin or glutaredoxin for the class Ia RNR (Table 1). Putative *nrdH* genes have been identified in the

²Oxidative stress can be regarded as a special case of iron limitation in which oxidation of Fur-bound Fe^{II} to Fe^{III} by reactive oxygen species leads to the derepression of Fur-regulated genes as a result of the lower affinity of Fur for Fe^{III} than for Fe^{II} (91).

genomes of most class Ib organisms. However, the annotated NrdHs of many *Bacillus* and *Staphylococcus* species have CXXC motifs (e.g., CPPC) distinct from those of most other NrdHs, and although *S. aureus* requires a class Ib RNR for aerobic growth, its NrdH is not essential in these conditions, making NrdH's role unclear (105). These organisms may use general reduction systems for RNR; for example, *Bacillus subtilis* has been proposed to use thioredoxin (106).

NrdI has been annotated as a flavodoxin-like protein (101) and is found in all organisms with genes for the class Ib RNR. *E. coli* NrdI was first isolated and characterized in 2008 (107); it is a 15-kDa monomer with a flavin mononucleotide (FMN) cofactor. On the basis of studies of YfaE and NrdB (70), this protein was initially hypothesized to play a role as the one-electron reductant required for biosynthesis or maintenance of an active $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor of NrdF (Figure 5a,b). The proposal was that, under iron-limited conditions in which the class Ib RNR was expressed, this flavodoxin would be a functional analog to a ferredoxin (YfaE). Indeed, NrdI in the fully reduced, hydroquinone (hq) form (NrdI_{hq} , containing FMNH^-) can stoichiometrically reduce met-NrdF to the diferrous state, allowing for reassembly of the $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor upon introduction of O_2 , supporting a maintenance function (107). Interestingly, in contrast to $[\text{2Fe2S}]^+\text{-YfaE}$ in class Ia, there is no evidence that NrdI_{hq} can serve as the source of the extra electron for class Ib $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ assembly, and in fact, it inhibits this process ($0.2 \text{ Y}\cdot/\beta 2$ versus $0.7 \text{ Y}\cdot/\beta 2$ in the absence of NrdI_{hq}) (31).

Further studies of NrdI, however, have demonstrated that it can behave as a two-electron reductant, more like a flavoprotein oxidase than a flavodoxin (31, 107). As discussed in more detail below, *E. coli* NrdF can form a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor in a self-assembly process requiring NrdI_{hq} and O_2 in vitro (Figure 5b) (31). This discovery led to the new proposal that NrdI_{hq} reacts with O_2 to provide the oxidant, H_2O_2 or HO_2^- [$\text{HOO}(\text{H})$], involved in biosynthesis of this dimanganese cofactor (31). $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ is very likely the active cofactor in most or all organisms containing a class Ib RNR, including *E. coli* (see below). The essentiality of NrdI for biosynthesis of this cofactor is probably general, and like YfaE for class Ia RNRs, NrdI may also be involved in cluster maintenance (Figure 5b).

Identity of the Metallocofactor of the Class Ib RNR

The history of efforts to identify the metallocofactor associated with the class Ib RNR has been recently summarized (31). In 1988, the RNR from *C. ammoniagenes* was the first class Ib enzyme to be purified and was proposed by Willing, Follmann, & Auling to require manganese for activity (30). Early studies with this organism showed that manganese depletion in growth media led to elongated cells in which protein and RNA synthesis were normal but DNA synthesis was impaired (108). Addition of Mn^{II} to the media resulted in a resumption of growth (108) and stimulation of RNR activity in cell extracts (30, 109). However, the purified RNR catalyzed nucleotide reduction at a very low rate (0.7 nmol/min/mg) (30), and no $\text{Y}\cdot$ was detectable by EPR spectroscopy (30, 110). Since those studies, the identity of the active cofactor in the class Ib RNRs has remained a matter of debate. The controversy arose and remained because an active $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor can be obtained in vitro by self-assembly with apo-NrdF, Fe^{II} , and O_2 (111), in analogy to the class

Ia RNRs (Figure 5a), or by overexpression of NrdF in a heterologous host (*E. coli*) (97). In fact, the amount and specific activity (SA) of the $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactors formed in *Salmonella* Typhimurium and *C. ammoniagenes* class Ib RNRs—SA of 850 nmol/min/mg and 1 $\text{Y}\cdot/\beta 2$ and 48 nmol/min/mg and 0.4 $\text{Y}\cdot/\beta 2$, respectively—were, until very recently, substantially higher than for any class Ib RNR isolated with manganese. SAs, $\text{Y}\cdot$, and metal contents for class Ib RNRs containing dimanganese or diiron cofactors characterized to date are summarized in Table 2. Furthermore, efforts to self-assemble an active dimanganese- $\text{Y}\cdot$ cofactor from apo-NrdF and Mn^{II} with a variety of oxidants were unsuccessful (111). However, very recent results have established that the *E. coli* and *C. ammoniagenes* NrdFs form a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor in vitro (31) and in vivo (32, 33).

Formation of an Active Dimanganese(III)- $\text{Y}\cdot$ Cofactor in NrdF In Vitro

Insight into the identity of the class Ib RNR cofactor and its mechanism of formation came from the discovery that *E. coli* NrdI has unusual redox properties (107) and forms a tight complex with NrdF (31). Most flavodoxins function as one-electron oxidants/reductants. Titration of their oxidized (ox) states with one-electron reductants, like sodium dithionite, result in stoichiometric amounts of a neutral semiquinone (sq) form of the FMN cofactor. The difference in reduction potential between the ox/sq and sq/hq couples is ~200–300 mV. When reductive titrations of NrdI were carried out, NrdI stabilized only 28% sq, requiring the reduction potentials of the two couples to be approximately equal. This property of NrdI and the possibility that manganese, not iron, is the metal in the class Ib RNR, suggested that NrdI might be involved in cluster biosynthesis, providing the oxidant, such as $\text{HOO}(\text{H})$, for oxidation of dimanganese(II) NrdF ($\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}$) to an active cofactor.

Assembly of an active dimanganese cofactor was therefore attempted in vitro by incubating $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}$ anaerobically with NrdI_{hq}, followed by exposure to O_2 . The resulting NrdF contained 0.25 $\text{Y}\cdot/\beta 2$ and exhibited an SA of 600 nmol/min/mg. The visible spectrum of the cofactor revealed characteristic features of a $\text{Y}\cdot$ (408 nm) and an oxidized manganese cluster (broad, trailing absorption features from 500 to 700 nm) (112). Extensive EPR analyses in comparison with model systems ruled out the formation of a mixed-valent ($\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ or $\text{Mn}^{\text{IV}}\text{Mn}^{\text{III}}$) cofactor and established that the $\text{Y}\cdot$ was responsible for the activity of the protein. The results demonstrated successful reconstitution of active $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor in NrdF.

The $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor could also be readily self-assembled from apo-NrdF, Fe^{II} , and O_2 , as previously reported for other class Ib RNRs, with no NrdI requirement. The dimanganese $\beta 2$ had higher activity than the diiron protein (600 versus 300 nmol/min/mg), even though its radical content was lower (0.25 versus 0.7 $\text{Y}\cdot/\beta 2$), and only ~40% of the total Mn^{II} (1.4 $\text{Mn}/\beta 2$) was oxidized in the assembly reaction. The low $\text{Y}\cdot$ content of the dimanganese enzyme and in complete cluster assembly could result, in the $\text{HOO}(\text{H})$ mechanism, from a requirement for additional components in the system, such as a NrdI reductase to facilitate the supply of the proposed 2 equiv (equivalents) of $\text{HOO}(\text{H})$ (see below) and for a source of the extra electron, both necessary for active cluster formation (Figure 5b, Scheme 3a).

The requirement of NrdI for $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor formation strongly suggested that NrdI_{hq} reacts with O_2 to produce the oxidant, H_2O_2 , HO_2^- , or $\text{O}_2^{\cdot-}$, required for Mn^{II}

oxidation. Control experiments with exogenous H_2O_2 and $\text{O}_2^{\bullet-}$ suggested that they were unlikely to be the oxidants, as they were unable to activate $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}$ in the absence of NrdI_{hq} or in the presence of NrdI_{ox} . HO_2^- was therefore proposed to be the oxidant. Furthermore, when reconstitution was carried out in the presence of catalase or SOD to scavenge HOO(H) or $\text{O}_2^{\bullet-}$, $\text{Y}^\bullet$ formation was not inhibited. This suggested that the oxidant generated by NrdI channels from its site of generation at the flavin to the metal site in NrdF within a $\text{NrdF}\cdot\text{NrdI}$ complex. Because HOO(H) is a two-electron oxidant, the stoichiometry of the overall reaction would require 2 equiv HOO(H) and an extra electron for $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}^\bullet$ cofactor assembly (Scheme 3a). A proposed mechanism for this process is shown schematically in Figure 5b. Because the identity of the oxidant in class Ib cluster assembly has not yet been determined, an alternative mechanism using $\text{O}_2^{\bullet-}$ as oxidant (Figure 5b) has also been proposed. The possibility of NrdI acting as a one-electron donor has been suggested by the observation that certain NrdI s stabilize much higher amounts of sq than the *E. coli* protein (see below) (49, 113, 114). This mechanism is attractive in that the reaction stoichiometry (Scheme 3b) requires one molecule of O_2 and one electron instead of two molecules of O_2 and five electrons if HOO(H) is the oxidant. Furthermore, oxidation of Mn^{II} to Mn^{III} by $\text{O}_2^{\bullet-}$ has precedent in manganese SODs (115), and production of a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ cluster in manganese catalases has been suggested to occur by oxidation of a $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ center by H_2O_2 on the basis of model chemistry (116, 117). Work to discriminate between these and other mechanistic possibilities is under way.

Structures of NrdF in Complex with NrdI

The involvement of a $\text{NrdF}\cdot\text{NrdI}$ complex in assembly of the class Ib metallocofactor is supported by the recent X-ray crystal structures of the $\text{NrdF}\cdot\text{NrdI}_{\text{ox}}$ and $\text{NrdF}\cdot\text{NrdI}_{\text{hq}}$ complexes (Figure 6a,b) (49). One NrdI binds per NrdF monomer, directly over a hydrophilic channel to the metal site. The hydrophilic nature of this channel differs from the hydrophobic nature of the channels that are proposed to function as access routes for O_2 to Fe^2 in the class Ia and Ic $\beta_2\text{s}$ (47, 48). The channel extends along the $\text{NrdI}\text{-NrdF}$ interface to the flavin cofactor (Figure 6b), consistent with oxidant delivery by tunneling. Additional support for the relevance of the channel in cluster assembly came from soaking crystals of $\text{NrdI}_{\text{ox}}\cdot\text{NrdF}$ with 100 mM sodium dithionite in the presence of O_2 . Strong additional electron density best modeled as a peroxide was observed in the channel near a constriction that may have to undergo a conformational change to allow the oxidant access to Mn^2 .

The $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ site also exhibits unique structural features relative to class Ia RNRs and to class Ib RNRs with Fe^{II} bound, a surprising result given that the protein residues involved in metal binding are identical (Figure 4). The unprecedented coordination of Glu158 in $\text{Mn}^{\text{II}}_2\text{-NrdF}$ (Figure 4) conformationally precludes formation of a hydrophobic pocket present in $\text{Fe}^{\text{II}}\text{Fe}^{\text{II}}$ forms of NrdB and NrdF , allowing O_2 to bind to Fe^2 . Furthermore, a unique water molecule coordinated to Mn^2 in $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}$ is proposed to dissociate and allow oxidant binding from the channel. The structural evidence thus suggests that the conformation of Glu158 controls the initial binding sites of the respective oxidants [O_2 and $\text{HOO(H)}/\text{O}_2^{\bullet-}$], depending on the metal bound to NrdF . Comparison of the reduced and oxidized forms of the dimanganese cofactor (Figure 4) reveals a basic principle: The conformational gymnastics of the carboxylates are required during the oxidation process.

An unresolved issue is the basis for the requirement for NrdI in $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor assembly in vitro. One possibility is that if the oxidant produced by NrdI_{hq} oxidation is HO_2^- its protonation state may be critical for diffusion to the metal site. The pK_a of H_2O_2 is 11.6, and thus insufficient concentrations of HO_2^- would be available in vivo. If $\text{O}_2^{\bullet-}$ is the oxidant, its production by NrdI and channeling to the metal site may be key to preventing its disproportionation before reaching Mn2. Alternatively, NrdI may induce a conformational change in NrdF to allow oxidant delivery to or reaction with the metal site. Binding of NrdI_{ox} or NrdI_{hq} induces no apparent conformational changes at the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ cluster in the crystal structures. However, the side chain of Ser154, a residue lining the channel near Mn2 and conserved only in class Ib RNRs, becomes ordered in a conformation linked by hydrogen bonding networks to both the bridging metal ligand Glu158 and to the water coordinated to Mn2. These networks may be crucial for Glu158's conformational mobility and ability to act as proton acceptor/donor during cluster oxidation (118, 119). The ligand reorganizations involved in the oxidation of $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}$ to the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor may be studied by addition of O_2 to NrdI_{hq}•NrdF crystals and monitoring structural changes.

NrdF: A Diiron or Dimanganese Protein In Vivo?

Although NrdF is catalytically active with both $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ and $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\bullet$ cofactors in vitro, recent results indicate that *C. ammoniagenes* (32), *E. coli* (33), and *B. subtilis* (Y. Zhang & J. Stubbe, in preparation) class Ib RNRs utilize $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactors in vivo. Auling, Lubitz, and coworkers have very recently demonstrated that overexpression of *C. ammoniagenes* NrdF to 5% of total cellular protein in its native organism with Mn^{II} in the growth medium leads to β_2 with a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor and an EPR spectrum identical to that observed in *E. coli* NrdF reconstituted in vitro using Mn^{II} , NrdI_{hq}, and O_2 (32, 120). The reported activity for dNDP formation, however, is very unusual, 69,000 nmol/min/mg, 10-fold higher than observed with any other class Ia, Ib, or Ic RNR, despite the fact that it contains only 0.36 Y•/ β_2 and 1.5 Mn/ β_2 (Table 2). This group has also recently reported the first structure (with 1.36-Å resolution) of an active manganese-loaded β_2 and its detailed characterization by multifrequency EPR methods (32). The EPR analysis suggests that the cluster contains a ferromagnetically coupled $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ cluster weakly coupled to the Y•. The overall structure of the cofactor is similar to the diferric cluster of the class Ia RNR (Figure 4). The weak ferromagnetic coupling and 3.3-Å Mn-Mn distance are consistent with μ -oxo, μ -carboxylato $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ model complexes (121), but the assignment of the oxidation states of the manganese ions in the structure is complicated by the possibility of photoreduction of the metal ions during data collection. This is a common problem in spectroscopy and crystallography of oxidized manganese centers, making detailed analysis of the structure challenging. Thus, we favor the possibility of a di- μ -hydroxo-bridged cluster with a shorter Mn-Mn distance in the active and/or met cofactors, in analogy to the manganese catalases (122, 123).

Our lab has recently purified NrdF expressed at endogenous levels (~0.03% of total protein) in iron-limited, Mn^{II} -supplemented growth conditions (33) from *E. coli* strain GR536 (124). This strain is deficient in the five currently known iron uptake systems. Purified NrdF contained 0.2 Y•/ β_2 and 0.9 Mn/ β_2 , and had an SA of 720 nmol/min/mg. Its EPR spectrum

is identical to that of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor reconstituted in vitro. Thus, NrdF likely uses a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor in *E. coli* and related enterobacteriaceae.

Finally, physiological studies in *B. subtilis*, which only contains a class Ib RNR, suggested that its NrdF is also manganese dependent. Direct evidence for this proposal has recently been obtained (Y. Zhang & J. Stubbe, in preparation) by purification of NrdF from a *B. subtilis* strain in which the levels of the class Ib operon proteins have been elevated 35-fold. Atomic absorption and EPR analyses demonstrate that NrdF contains a dimanganese cluster similar to the *E. coli* and *C. ammoniagenes* enzymes. Interestingly, the *B. subtilis* class Ib RNR operon (*nrdIEF*) also contains a fourth gene, *ymaB*, of unknown function (125).

Whether all NrdFs use dimanganese cofactors in vivo remains to be established and requires purification from their respective organisms. However, in the absence of this additional information, one may be able to predict which NrdFs require manganese by understanding the redox properties of *E. coli* NrdI that make it effective in $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cluster assembly. One feature of NrdI proteins likely to have a significant effect on the FMN redox potentials is the interaction of the flavin with a conserved loop, designated the “50s loop,” which is located structurally adjacent to the C4a position of the flavin, which reacts with O_2 (33, 49, 107). Phylogenetic analyses show three main branches of NrdIs, classified by the length and composition of this loop.

NrdIs of the major branch, which includes the *C. ammoniagenes* and *E. coli* class Ib RNRs, contain a Gly-rich 50s loop of ~7 residues. These NrdIs are found in enterobacteriaceae, corynebacteriaceae, mycobacteriaceae, and *Streptococcus* [including *S. pyogenes*, in which one of its NrdIs (designated NrdI*) has been shown to be essential (101)]. These NrdIs are expected to have redox properties similar to *E. coli* NrdI and to play a similar role in O_2 activation, and their associated NrdFs will likely use dimanganese cofactors in vivo.

A second, smaller class contains NrdIs from *Lactobacillus*, *Lactococcus*, and *Enterococcus*, as well as the nonessential NrdI2 of *S. pyogenes*. These phylogenetically grouped NrdIs have either ~7-residue 50s loops lacking Gly or longer (~14-residue) loops with Gly residues. This category includes *Lactobacillus plantarum*, an organism that accumulates high levels of manganese and does not require iron for growth; its class Ib RNR is therefore expected to use a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\bullet$ cofactor.

NrdIs of the third branch have short three-residue 50s loops, usually Gly-Phe-Gly, and are found mainly in *Bacillus* and *Staphylococcus*. The class Ib RNRs of phytoplasmas, obligate intracellular plant pathogens, also fall into this group; in these cases, NrdI does not exist as a separate protein but is instead fused to the N terminus of NrdE. Crystal structures and redox titration experiments of NrdIs from *B. subtilis* (R. Wu, R. Zhang, F. Collart & A. Joachimiak, unpublished results, Protein Data Bank code 1RLJ), *Bacillus cereus* (113), and *B. anthracis* (114) have been reported. *B. subtilis* and *B. cereus* NrdIs share 48% sequence identity. Surprisingly, although the redox properties of *B. subtilis* NrdI (Y. Zhang & J. Stubbe, in preparation) are similar to those of *E. coli* NrdI, reductive titration of *B. cereus* NrdI demonstrates that it stabilizes nearly stoichiometric amounts of flavin sq (113), suggestive of a function involving one-electron chemistry. Whereas *B. subtilis* NrdF

contains a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cluster, purified NrdF from *B. cereus* has not yet been reported. The redox properties of *B. cereus* NrdI suggest that it may function as a one-electron reductant for assembly or maintenance of a $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor or that a different reduced O_2 species (e.g., $\text{O}_2^{\cdot-}$) may be involved in $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor assembly. Alternatively, the redox properties of *B. cereus* NrdI could be modulated by NrdF or other protein factors necessary for cluster assembly to function as a two-electron reductant. It remains to be determined whether the manganese dependency of *B. subtilis* RNR is the exception or the norm for this branch of class Ib RNRs.

The available evidence so far in a diverse set of organisms (*E. coli*, *C. ammoniagenes*, and *B. subtilis*) has demonstrated formation of a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor in the class Ib RNR in vivo. The details of the growth conditions from which these dimanganese NrdFs were purified suggest that manganese is preferentially loaded into NrdF even in the presence of iron in the growth media, at least in *C. ammoniagenes* and *B. subtilis*. Although the presence of a $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor in other organisms cannot be completely excluded, no in vivo evidence exists for this possibility at present.

CLASS Ic RIBONUCLEOTIDE REDUCTASES

As with class Ib, the role of manganese in the active metallocofactor of the class Ic RNRs was not appreciated at first. Initial studies led to the proposal that the active enzyme used a $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor in place of a $\text{Y}\cdot$. Subsequent work has cast doubt on the activity of this cofactor, whereas the protein can clearly utilize a $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor for nucleotide reduction. The ability of this cofactor to form in vivo, however, has not yet been probed.

Discovery of an Active $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ Cofactor

The class Ic RNR was discovered by McClarty and coworkers in *Chlamydia trachomatis* (126), an obligate intracellular pathogen. Determination of its sequence and comparison with that of other RNRs (88) also suggest the presence of class Ic RNRs in the genomes of certain archaea and eubacteria (127, 128). Sequence alignments reveal that all the residues in the PCET pathway (Figure 3) and active site for nucleotide reduction are conserved. However, important differences at and adjacent to metal site 1 of β_2 (NrdB) relative to the class Ia and Ib RNRs are apparent (Figure 1b). First, Phe127 in *C. trachomatis* NrdB aligns with the Tyr oxidized to the $\text{Y}\cdot$ in the class Ia and Ib RNRs (Figure 4). Second, Glu89 (*C. trachomatis* NrdB) replaces Asp84 (*E. coli* NrdB), a ligand to Fe1. In early studies (126) in which recombinant *C. trachomatis* RNR expressed in and isolated from *E. coli* was incubated with HU, RNR activity was drastically reduced, suggesting the importance of a $\text{Y}\cdot$ in catalysis. It was initially proposed that Tyr129 could be the site of $\text{Y}\cdot$ formation, but the X-ray crystal structure (127) confirmed that Phe127 is located in a position very similar to the Tyr oxidized in class Ia and Ib RNRs (Figure 4). Tyr129, by contrast, resides on the protein surface. The structure also revealed a diferric cluster with a terminal H_2O and two bridging H_xO ligands assigned as hydroxides (Figure 4), similar to the diferric cluster of soluble methane monooxygenase (129) and distinct from all class Ia and Ib cluster structures to date.

Initial attempts to self-assemble the active cofactor for *C. trachomatis* NrdB starting with Fe^{II} and O_2 revealed an EPR-active species similar to intermediate **X** (Figure 5a), the

oxidant required for Tyr oxidation in class Ia cofactor assembly. Thus, the hypothesis became that, in the absence of a nearby Tyr to be oxidized, the $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ species (**X**) replaces the $\text{Y}^\bullet$ as the active radical initiator in class Ic RNRs (127). Although this hypothesis was at first intriguing, a number of issues became apparent when examining the data in detail. First, the SA reported for this class Ic RNR was low and variable (130, 131). It should be noted, however, that the rate of dNDP formation required to meet the needs of *Chlamydia* for DNA replication is not currently known. Second, recent studies failed to observe a correlation between RNR activity and levels of **X** (34). This is in contrast to the class Ia and Ib enzymes, where the activity correlates with the concentrations of the $\text{Y}^\bullet$ (31, 132), suggesting that **X** is not the active cofactor. Third, and problematic from a chemical perspective, is the questionable ability of a pathway residue, such as Tyr338 or Trp51 (equivalent to Tyr356 or Trp48 in *E. coli* NrdB, Figure 3), to reoxidize *C. trachomatis* $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}$ -NrdB to $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ at the end of each catalytic cycle. Our recent studies of the *E. coli* class Ia RNR with an unnatural amino acid, 3-nitrotyrosine (NO_2 Y), site specifically replacing Tyr122, have shown that intermediate **X** can oxidize a NO_2 Y to a NO_2 $\text{Y}^\bullet$ despite the fact that an *N*-acylated, esterified NO_2 Y amino acid is 200 mV more difficult to oxidize than a similarly blocked Tyr (44). Thus, the ability of a Tyr or Trp radical in the PCET pathway to reoxidize a $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}$ cluster to $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ seems unlikely (44, 133). A recent density functional theory study has come to the same conclusion (134). However, the possibility that the class Ic RNRs catalyze only a single turnover, with the cofactor regenerated by a specific repair pathway or de novo biosynthetic pathway for every dNDP produced (131), cannot be ruled out.

The variability of *C. trachomatis* RNR activity, the lack of correlation of activity with iron content, and the inability to replicate the published generation of an active cofactor with Fe^{II} and O_2 (130) suggested to the Bollinger and Krebs laboratories (34) that the metallocofactor of *C. trachomatis* $\beta 2$ had been misidentified. Their careful studies³ demonstrated that $\beta 2$ activity is dependent on the presence of both manganese and iron, in an unprecedented $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor (described below), and that its SA is ~ 600 nmol/min/mg when apoprotein is reconstituted with 2 Mn^{II} and 2 $\text{Fe}^{\text{II}}/\beta 2$ in the presence of O_2 (34). The lower activities reported earlier (130, 131) were suggested to have arisen from variable amounts of undetected, “contaminating” manganese (34). More recent studies have found that an ordered loading of $\beta 2$ under aerobic conditions with 3 $\text{Mn}^{\text{II}}/\beta 2$ prior to addition of 1.5 $\text{Fe}^{\text{II}}/\beta 2$ maximizes $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ and minimizes $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ production (still 10% of metal sites) (135). Although the $\text{Mn}^{\text{II}} \text{Mn}^{\text{II}}$ form of the protein does not react with O_2 , the $\text{Fe}^{\text{II}}\text{Fe}^{\text{II}}$ form does [$k = 2.8 \text{ s}^{-1}$ for **X** formation (130)], raising the issue of how mismetallation is prevented in vivo with this cofactor.

Mechanism of $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ Cofactor Assembly In Vitro

The proposed structure and basic mechanism of formation of the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor (Figure 5c) have been recently and extensively reviewed (136). We focus here on the mechanism of

³Nearly all studies of the *C. trachomatis* RNR have used an $\alpha 2$ subunit in which the N-terminal 248 residues have been truncated to remove two of the three ATP cone domains. Now that the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor has been discovered and detailed studies of PCET are being carried out (45), the early studies justifying use of this truncated protein (126) should be revisited to determine whether the truncation affects conformational gating of PCET, allosteric regulation of nucleotide reduction, and $\alpha 2\beta 2$ interaction.

its self-assembly in vitro in relation to those of the class Ia and Ib metallocofactors and the issues raised by this information for cluster assembly in vivo.

In vitro self-assembly was optimized to give 1.5 Mn^{IV}Fe^{III}/β2 by ordered addition of Mn^{II} and Fe^{II}, as described above (135). Mössbauer and EPR spectroscopies were used to characterize this cofactor and reveal the mechanisms of its formation. The active cofactor contains Mn^{IV} and Fe^{III} ions antiferromagnetically coupled with an $S = 1$ ground state (34, 137). The structure of this cofactor has not yet been established crystallographically, but EXAFS analysis and density functional theory calculations (135) suggest μ-oxo, μ-hydroxo, and μ-1,3-carboxylato bridges between the metals, which are ~2.92 Å apart (Figure 5c). Although the calculations cannot establish the site of manganese binding, site 1 is generally favored (136) on the basis of the Asp to Glu substitution and the observation of manganese in this site in the crystal structure of a manganese-iron oxidase from *M. tuberculosis* (see sidebar, Manganese-Iron Oxidases) (138). As Mn^{IV}Fe^{III} assembly is maximized by adding Mn^{II} before Fe^{II}, this proposal implies that site 1 is the higher-affinity metal site in *C. trachomatis* NrdB, which contrasts with *E. coli* NrdB where site 2 has higher affinity for both Fe^{II} and Mn^{II} (63, 139–141). This difference might be accounted for in part by the Asp to Glu substitution at site 1, which would make the ligand environments of the two sites more similar. The location of redox-inert Phe127 (Phe, Leu, Ile, or Val in other predicted class Ic RNRs) adjacent to site 1 is proposed to create a stabilizing environment for Mn^{IV} (128).

As in class Ia, cofactor self-assembly in the class Ic RNR has been studied by SF-UV-vis spectroscopy and by RFQ EPR and Mössbauer spectroscopies. Rapid mixing of *C. trachomatis* Mn^{II}Fe^{II}-NrdB with O₂ results in the formation of a Mn^{IV}Fe^{IV} intermediate ($k = 13,000 \text{ M}^{-1} \text{ s}^{-1}$) with an $S = 1/2$ ground state arising from anti-ferromagnetic coupling between the two metal sites (142). This intermediate can be slowly reduced ($k_{\text{obs}} = 0.021 \text{ s}^{-1}$) to the active Mn^{IV}Fe^{III} (Figure 4) (143). This reduction step has been proposed to proceed by a two-step pathway through Trp51 (equivalent to Trp48 in *E. coli* NrdB) and Tyr222, instead of the equivalent residue to *E. coli* NrdB Tyr356 (Tyr338) (68). Tyr222 is conserved uniquely in class Ic β2s, but it is not essential for cluster assembly or for the PCET pathway (143).

The four oxidizing equivalents required to form the Mn^{IV}Fe^{IV} intermediate from Mn^{II}Fe^{II}-NrdB can also be provided by 2 equiv of H₂O₂ and a stepwise mechanism. The first equivalent oxidizes the Mn^{II}Fe^{II} cluster to the Mn^{III}Fe^{III} state (k of $1,700 \text{ M}^{-1} \text{ s}^{-1}$ for Mn^{III}Fe^{III} formation), which can react with a second equivalent of H₂O₂ ($k = 8 \text{ M}^{-1} \text{ s}^{-1}$) to generate the Mn^{IV}Fe^{IV} state. This state then decays to the active Mn^{IV}Fe^{III} cofactor as discussed above (144). The physiological assembly pathway is unclear.

To summarize cluster assembly in the class I RNRs (Figure 5), the Fe^{II}Fe^{II} forms of the class Ia and Ib enzymes and the Mn^{II}Fe^{II} form of class Ic can all react with O₂ and a reductant to form active Fe^{III}Fe^{III}-Y• and Mn^{IV}Fe^{III} cofactors, respectively. The class Ic Mn^{II}Fe^{II}-NrdB, on the one hand, can also react with 2 equiv of H₂O₂ to form active cofactor. On the other hand, the class Ia Fe^{II}Fe^{II}-NrdB reacts with H₂O₂ to produce the inactive met Fe^{III}Fe^{III} cluster, but further oxidation occurs very slowly and to a limited

extent (145). In contrast to the class Ia and Ic RNRs, the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ form of class Ib RNRs cannot be activated by either exogenous O_2 or H_2O_2 (18, 31, 111). Another protein, NrdI_{hq} , is essential, and depending on the mechanism of $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ assembly, it is possible that it is required only to reduce O_2 to form the oxidant for cluster formation (Scheme 3, Figure 5b).

Are Both $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ and $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ Cofactors Active?

The arguments enumerated above suggest that the original proposal that the $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor is active in dNDP formation is incorrect (127, 130, 131). The strongest evidence against this proposal comes from studies with *C. trachomatis* $\beta 2$ containing either a $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ or $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor, $\alpha 2$, and the mechanism-based inhibitor 2'-azido-2'-deoxyadenosine 5'-diphosphate (N_3ADP). Previous studies with the class Ia RNRs indicate that the $\text{Y}\cdot$ is reduced concomitant with formation of a well-characterized nitrogen-centered radical derived from breakdown of N_3ADP (146). However, in the case of *C. trachomatis* $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ - NrdB , addition of N_3ADP under turnover conditions did not accelerate decay of the X-like EPR signal, suggesting that the $\text{Fe}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor is not competent for nucleotide reduction, in contrast to similar studies with $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ - NrdB (34). Therefore, the current evidence supports only the activity of the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor. At present, *E. coli* and *C. ammoniagenes* NrdF s are the only examples of RNRs with high catalytic activity with two different metallocofactors.

Relevance of the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ Cofactor In Vivo

It was initially proposed (127) that the $\text{Y}\cdot$ -less class Ic RNRs might have evolved in pathogenic organisms as a mechanism of resistance to $\text{O}_2^{\bullet-}$, NO, and peroxynitrite, oxidants produced by a host's immune system and known to react with the $\text{Y}\cdot$ of *E. coli* NrdB (147, 148). The details of these reactions deserve further study, and the reactivity of the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor with these species has not yet been reported. Furthermore, the hypothesis also needs to be examined in light of the observation that many pathogens use class Ib RNRs and a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor. The stability of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor in the presence of $\text{O}_2^{\bullet-}$ and NO is also not currently known, but it is possible that the class Ib and Ic RNR cofactors may constitute different solutions to the same problem of oxidative stress for certain pathogenic organisms.

Given the complexities of heterobinuclear cluster formation in vitro (see above) and in vivo (see below), as well as the high catalytic activity of the class Ib RNR containing two different cofactors, it may be premature to conclude that the $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ is the physiologically relevant cofactor of the class Ic RNRs. It is therefore essential to isolate *C. trachomatis* NrdB from the native organism to demonstrate whether this, or another cofactor such as $\text{Mn}^{\text{IV}}\text{Mn}^{\text{III}}$, is the active form of the class Ic RNR in vivo.

CONCLUSION: METALLATION OF THE CLASS I RNRs IN VIVO

How metalloproteins acquire their correct metals and avoid mismetallation inside the cell is a major unsolved problem of bioinorganic chemistry. A key issue is that the metal required for activity by a given metalloprotein is often not the metal that binds to that protein with the

highest affinity. Although metal affinity is in part dependent on the protein coordination environment, the relative affinities of proteins for divalent metals can generally be described by the Irving-Williams series ($\text{Mn}^{\text{II}} < \text{Fe}^{\text{II}} < \text{Co}^{\text{II}} < \text{Ni}^{\text{II}} < \text{Cu}^{\text{II}} > \text{Zn}^{\text{II}}$) (149). Interestingly, metal chaperones have been identified only for metals late in this series (cobalt, nickel, copper, zinc) and the “free” intracellular concentrations of these metals are extremely low (1, 14). One way to overcome the binding preference of a protein for the incorrect metal is cellular compartmentalization. Tottey et al. (15) have recently demonstrated in cyanobacteria that a Mn^{II} -dependent periplasmic protein must fold in the cytosol, where levels of Mn^{II} are much higher than levels of free Cu^{I} , Cu^{II} , and Zn^{II} , to ensure correct metallation. In fact, work from many groups reveals that chaperone proteins exist not only for correct metal delivery of copper, zinc, nickel, and cobalt, but also to maintain very low free concentrations of these metals, which prevents their binding to other proteins that require metals early in the series, such as iron and manganese.

Iron, present at high concentrations in most organisms (e.g., $\sim 100 \mu\text{M}$ for *E. coli*) (14) in complex with nucleic acids, proteins, or small-molecule chelators, may also require a protein or small-molecule chaperone to limit the deleterious chemistry associated with the reaction of Fe^{II} with O_2 or H_2O_2 and the insolubility of the resulting Fe^{III} . Unlike Fe^{II} , Mn^{II} does not seem to react with O_2 and H_2O_2 ⁴ under physiological conditions, and thus, free manganese can exist in cells, complexed with small molecules (phosphate, nucleotides) or nucleic acids, without having to worry about oxidative damage under physiological conditions. Manganese is present at lower concentrations than iron in *E. coli* [1 to $10 \mu\text{M}$, depending on growth conditions (14)], but some organisms accumulate far higher concentrations millimolar, in *L. plantarum* (151)]. In the former, Mn^{II} has been shown to play a key role in oxidative stress resistance (17). In the latter organisms, the exceedingly high free Mn^{II} concentration is important in oxidative defense and is proposed to replace the function of SODs, which these organisms lack (151); Mn^{II} phosphate or pyrophosphate complexes have been shown in vitro to act as a SOD (152). Recent whole-cell ENDOR spectroscopic studies of yeast cells have also supported this role (153). Two classes of SODs catalyze the oxidation of $\text{O}_2^{\bullet -}$ to O_2 and H_2O_2 using either a manganese or iron cofactor. Culotta and coworkers (154) have proposed that correct metallation of *S. cerevisiae* manganese SOD with Mn^{II} rather than Fe^{II} relies on the “differential bioavailability” and relative affinities of the protein for the two metals, rather than on a Mn^{II} chaperone protein. Mn^{II} concentrations are high relative to other redox-active metals, Mn^{II} does not contribute to oxidative stress (and in fact counteracts it), and Mn^{II} is low in the Irving-Williams series. For these reasons, chaperone proteins may not exist for Mn^{II} delivery to the relatively few enzymes that require it. These observations have important implications for metal loading of the class Ib and Ic RNRs.

Each class I RNR presents a unique problem with respect to correct metal loading. The class Ia RNR uses a $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor, but it binds Mn^{II} more tightly than Fe^{II} (18). Correct metallation of *E. coli* NrdB may hinge, therefore, on the relative concentrations of free Fe^{II} and Mn^{II} in the cell or on overcoming the protein’s preference for Mn^{II} by using a specific iron chaperone, our favored model. Proteins involved in delivering iron to ferritin (155) and

⁴Although bicarbonate complexes of Mn^{II} decompose H_2O_2 (150), studies in *E. coli* (17) suggest that intracellular Mn^{II} , which accumulates in oxidative stress, is not an important scavenger of H_2O_2 in these conditions.

liberating iron from bacterioferritin (156) have been identified, and the highly conserved protein frataxin has also been proposed to be involved in delivery of iron in FeS cluster biogenesis (reviewed in Reference 157). As discussed above, evidence also exists for labile FeS clusters as iron trafficking agents in the cell. In general, however, establishing definitive links between iron-dependent proteins and their sources of iron in trafficking pathways has been challenging owing to the chemical properties of Fe^{II}, the redundancy of iron transport systems, and the difficulty of monitoring iron movement spectroscopically. A combination of biochemistry, spectroscopy, and genetics, as well as new methods of analysis, will lead the way in obtaining a deeper understanding of iron delivery in vivo.

For reasons articulated above, we postulate that the class Ib RNRs of most or all organisms use a Mn^{III}Mn^{III}-Y• cofactor. In *E. coli*, this protein is induced when iron is not readily bioavailable or cells experience oxidative stress. However, many other organisms, including pathogens, encounter these conditions on a regular basis and require a class Ib RNR. This distinction may be related to variations in metal homeostatic mechanisms and thus in the resulting concentrations of accessible Mn^{II} and Fe^{II} in different organisms. For pathogens, the added complexity of the relative affinities and specificities of transporters for Mn^{II}, Fe^{II}, and other divalent metals between pathogen and host also needs to be considered (158).

Unfortunately, little information is available about the intracellular concentrations of various metals in many organisms and how those concentrations change with growth conditions. However, the affinities of metal transport regulators for a specific metal may be used as a proxy for approximate free metal concentrations in cells (159). The *B. subtilis* Mn^{II} transport regulator MntR has low affinity for Mn^{II} (~160 μM) (160), suggesting that this organism may contain higher intracellular concentrations of free Mn^{II} than does *E. coli*. The lower levels of Mn^{II} in *E. coli* versus *B. subtilis* and perhaps many pathogens may be closely linked to why *E. coli* primarily uses an iron-dependent class Ia RNR, whereas *B. subtilis* and many other prokaryotes use a manganese-dependent class Ib RNR. If a select group of class Ib RNRs is found to use an Fe^{III}Fe^{III}-Y• cofactor, as discussed above, this organism-dependent variation in metal homeostasis may provide an explanation.

Finally, the presence of a Mn^{IV}Fe^{III} cofactor in the class Ic RNR if verified in vivo poses an additional complication for assembly, the requirement to insert two different metals with similar affinities for the protein. In vitro studies with *C. trachomatis* NrdB have demonstrated the importance of controlling the amounts and the order of addition of the metals (31, 142). If Mn^{II} is absent or present at an inappropriate ratio relative to Fe^{II}, for example, inactive diiron clusters can be formed. Additionally, mismetallation and low amounts of active cofactor are the results of the inability to control the availabilities of manganese and iron when protein is overproduced in *E. coli* in rich media.

A few strategies can be envisioned to have evolved to generate the Mn^{II}Fe^{II} cluster required for assembly of the active cofactor in the presence of O₂ in vivo. The free Mn^{II} concentrations in chlamydiae might be higher than free Fe^{II} concentrations, with iron limitation the result of the host's immune response. If β₂ is first loaded to form an unreactive Mn^{II}Mn^{II} cluster and binding of the second metal is relatively weak, as is the case in the class Ia RNRs, then exchange of the second Mn^{II} with Fe^{II} might occur (J.M.

Bollinger, Jr., personal communication). Alternatively, Fe^{II} loading may be aided by an iron chaperone, once Mn^{II} is loaded into the high-affinity site. The small size of the *C. trachomatis* genome (1 million bp) could be a boon for studies aimed at identifying such a factor, as fewer genes would suggest fewer redundancies in metal delivery pathways, and the host organism would not be expected to contain such a factor. The knowledge gleaned from this relatively simple system might then be the key to unlock the complexities of metal delivery in other organisms.

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Glossary

RNR	ribonucleotide reductase
SOD	superoxide dismutase
EPR	electron paramagnetic resonance
Y•	tyrosyl radical, essential for catalysis in the class Ia and Ib ribonucleotide reductases
β2	ribonucleotide reductase small subunit, containing the metallocofactor essential for catalysis
PCET	proton-coupled electron transfer
NrdB	the β2 subunit of the <i>E. coli</i> class Ia and <i>C. trachomatis</i> class Ic ribonucleotide reductases
α2	ribonucleotide reductase large subunit, containing the site of nucleotide reduction
ENDOR	electron-nuclear double resonance
EXAFS	extended X-ray absorption fine structure
Intermediate X	an Fe ^{IV} Fe ^{III} species that is the direct precursor to the Fe ^{III} Fe ^{III} -Y• cofactor in the class Ia RNR assembly pathway
YfaE	a ferredoxin proposed to be involved in the biosynthetic and maintenance pathways of the <i>E. coli</i> class Ia ribonucleotide reductase
HU	hydroxyurea
NrdF	the β2 subunit of the class Ib ribonucleotide reductases
NrdI	a flavoprotein proposed to react with O ₂ to provide the oxidant required for Mn ^{III} Mn ^{III} -Y• assembly in the class Ib ribonucleotide reductases

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MANGANESE-IRON OXIDASES

Recently Högbom (128) has carried out extensive sequence alignments with class Ic NrdB in search of other proteins utilizing manganese-iron cofactors. *M. tuberculosis* Rv0233 was identified and overexpressed in *E. coli* in rich medium. Unlike *C. trachomatis* NrdB expressed under similar conditions, it contained approximately equal amounts of manganese and iron, confirmed by metal analysis and a crystal structure at 1.9-Å resolution (138). Anomalous diffraction data indicated that manganese was located at site 1 and iron at site 2 despite the symmetric arrangement of the coordinating ligands. Rv0233 contains the same metal coordinating residues as class Ic RNR but in addition contains an unprecedented tyrosine-valine cross-link and a lipid with its carboxylate moiety coordinated to both the manganese and the iron. The function of this protein and others identified by the sequence analysis remains unknown. Further biochemical and biophysical studies on this system and other systems will hopefully contribute to our understanding of metal ion specificity and reaction mechanisms in RNRs and related proteins.

SUMMARY POINTS

1. The assembly of metallocofactors often requires specialized metal insertion machinery and the involvement of accessory proteins. Physiological expression conditions must be considered in studies of metalloenzymes to ensure that the form of the protein being studied in vitro is the active form in vivo.
2. Ribonucleotide reductases (RNRs) catalyze the reduction of the four nucleotides to their corresponding deoxynucleotides and thus play an essential role in DNA replication and repair pathways. RNRs are classified by the five different metallocofactors involved in the formation of a transient thiyl radical that initiates catalysis.
3. The class Ia RNRs use a $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor, whose mechanism of formation has been studied extensively in vitro. In vivo a $[2\text{Fe}2\text{S}]$ ferredoxin, YfaE, is likely involved in *E. coli* class Ia RNR cofactor assembly as the source of the “extra” electron, as well as in the maintenance pathway.
4. The *E. coli* class Ib RNR is active in vitro with both $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactors. However, only the dimanganese cofactor has been found so far to be relevant in vivo. Formation of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor is proposed to occur via oxidation of a $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ center by an oxidant produced by reduction of O_2 by a conserved flavoprotein, NrdI.
5. In contrast to the other class I RNRs, the class Ic RNRs store the one-electron oxidizing equivalent in the metal cluster and are active using a $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor. No class Ic RNR has been isolated to date from its native organism.
6. Evidence suggests the presence of a maintenance pathway by which an active cofactor can be regenerated from an inactive cofactor. This maintenance pathway will be important for understanding the activity and regulation of RNRs and, in humans, the mechanism of chemotherapeutics that target $\text{Y}\cdot$ reduction.

FUTURE ISSUES

1. What are the mechanisms of metal delivery to RNRs? The class Ia, Ib, and Ic RNRs have similar metal coordination environments but use different cofactors. How is correct metallation controlled?
2. What is the ultimate source of the extra electron involved in cluster assembly?
3. What factors are involved in cofactor maintenance in other RNR systems? Mammalian systems are of particular interest because of the instability of the $Y\bullet$.
4. Do all class Ib RNRs use a $Mn^{III}Mn^{III}-Y\bullet$ cofactor? What is the mechanism of assembly of this cofactor?
5. Do class Ic RNRs expressed at endogenous levels and isolated from their native organism contain the $Mn^{IV}Fe^{III}$ cofactor? If so, how is correct metallation controlled?
6. Why has nature evolved three different metallocofactors for class I RNRs?
7. Do chaperone proteins exist for iron and manganese?
8. Are the lessons learned from RNRs generally applicable to metal delivery to nonheme diiron proteins and mononuclear iron and manganese proteins (e.g., SOD)?

Figure 1.

(a) Structures and (b) metallocofactors of the class I RNR $\beta 2$ subunits. (a) The class Ia *E. coli* NrdB (Protein Data Bank code: 1MXR) (161), the class Ib *E. coli* NrdF (3N37) (49), and the class Ic *Chlamydia trachomatis* NrdB (1SYY) (127). Fe^{III} and Mn^{II} ions are shown as brown and purple spheres. Images were generated using PyMOL. (b) The metals and protein residues involved in metal binding are shown in cartoon form. More detailed structures are shown in Figure 4. The metal site closest to Tyr or Phe is termed site 1 (Mn1, Fe1) and the other is site 2 (Mn2, Fe2). Because the coordination modes of the Asp and Glu residues are dependent on the oxidation state of the cluster, no metal-ligand bonds are drawn. The structure of the class Ia $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$ cofactor has been established. The class Ib $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ has been crystallized (32); however, owing to the sensitivity of manganese to photoreduction, the oxidation states of the manganese ions and the number and identity of bridging ligands are not clear. The $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ cofactor of the class Ic cluster has not been crystallographically characterized, and in the structure shown, the proposed placements of manganese and iron are based on extended X-ray absorption fine structure (EXAFS) data (135).

Figure 2.

Proposed mechanism of nucleotide reduction by ribonucleotide reductases (RNRs). The active sites of all three classes of RNRs share a conserved cysteine (Cys) residue (SH) on the top face of the substrate. In the first step of catalysis, this cysteine is oxidized to a thiyl radical ($S^\bullet$) by a tyrosyl radical ($Y^\bullet$; class Ia, Ib), $Mn^{IV}Fe^{III}$ cofactor (class Ic), 5'-deoxyadenosyl radical (class II), or a glycy radical (class III). The $S^\bullet$ initiates substrate reduction by abstraction of the nucleotide's 3' hydrogen atom (*red*), which is returned to the 3' position in the product at the end of the reaction. For a detailed discussion of this

mechanism, see Reference 39. In class I and II RNRs, two Cys residues located on the bottom face of the substrate are the direct source of the reducing equivalents for nucleotide reduction. Reduction of the resulting disulfide bond, necessary for multiple turnovers, is accomplished using electrons from a thiol-dependent protein (thioredoxin or glutaredoxin). Class III RNRs differ from classes I and II in that only one Cys residue on the bottom face is conserved, and formate acts as the reductant. Class III RNRs also lack the Glu ($-\text{CO}_2^-$) and Asn ($-\text{CONH}_2$) residues conserved in the active sites of class I and II RNRs.

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Figure 3.

The proposed proton-coupled electron transfer (PCET) pathway of all class I ribonucleotide reductases (*E. coli* class Ia numbering is used). PCET is triggered by binding of substrate and effector to α . In β , proton transfers are proposed to move orthogonally to electron transfers, while in α , they are proposed to move collinearly. The proton donor/acceptor for Tyr122 is proposed to be the solvent molecule bound to Fe1, with proton transfer being mediated by Asp84. Glu350 and Tyr356 are located in the C terminus of β , which is not observed in any structures; therefore, the connection of the PCET pathway across the subunit interface is unknown at present.

Figure 4.

Structures of the reduced (*left*) and oxidized (*right*) metallocofactors of the class I ribonucleotide reductases. Solvent molecules are shown as red spheres, and iron and manganese ions are brown and purple spheres. The images were generated using PyMOL from the following Protein Data Bank files: *E. coli* Fe^{II}Fe^{II}-NrdB (1PIY) (162), *E. coli* Fe^{III}Fe^{III}-NrdB (1MXR) (161), *E. coli* Mn^{II}Mn^{II}-NrdF (3N37) (49), *Corynebacterium ammoniagenes* Mn^{III}Mn^{III}-NrdF (3MJO) (32), *E. coli* Fe^{II}Fe^{II}-NrdF (3N38) (49),

Salmonella enterica serovar Typhimurium Fe^{III}Fe^{III}-NrdF (2R2F) (96), and *C. trachomatis* Fe^{III}Fe^{III}-NrdB (1SYY) (127).

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Figure 5. The proposed biosynthetic and maintenance pathways for the metallocofactors of the class Ia (a), Ib (b), and Ic (c) ribonucleotide reductases. The steps shown in blue highlight the requirement for the extra reducing equivalent, and in red, the maintenance pathway. In the case of class Ib, two possible routes to the putative $\text{Mn}^{\text{IV}} \text{Mn}^{\text{III}}$ intermediate are shown. In black is the mechanism originally proposed (31) in which NrdI acts as a source of $\text{HOO}(\text{H})$. In green is an alternative mechanism in which NrdI acts as a source of $\text{O}_2^{\bullet -}$. In this mechanism, either NrdI_{hq} or NrdI_{sq} might serve as the one-electron reductant of O_2 ; a

distinction cannot be made between these possibilities at this time, and only the former is shown. Details of the proposed mechanisms are described in the text.

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Figure 6.

Structure of the *E. coli* $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}\cdot\text{NrdI}_{\text{ox}}$ complex (a) with an enlarged view of the channel that connects the flavin in NrdI to $\text{Mn}2$ in NrdF (b). (a) The $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}\text{-NrdF}\cdot\text{NrdI}_{\text{ox}}$ complex with NrdF in gray, NrdI_{ox} in green, Mn^{II} in purple spheres, and flavin mononucleotide (FMN) in sticks. (b) Binding of NrdI to NrdF extends a hydrophilic channel (blue mesh) for the oxidant generated in NrdI to access $\text{Mn}2$. The residues shown in sticks lining the channel are highly or completely conserved in NrdI s and NrdF s. Reproduced with permission from Reference 49.

Scheme 1.

The reaction catalyzed by ribonucleotide reductases (RNRs). Class I RNRs use only nucleoside 5'-diphosphate substrates. PPO denotes pyrophosphate ($P_2O_7^{2-}$). The reaction is initiated by abstraction of the 3' hydrogen atom (*red*). Modified from Reference 28.

Scheme 2.

Stoichiometry of diferric-tyrosyl radical ($\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$) cofactor formation.

Scheme 3.

Proposed stoichiometries of $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}\text{-Y}\cdot$ cofactor formation with (a) $\text{HOO}(\text{H})$ and (b) $\text{O}_2^{\cdot-}$ as oxidant.

Table 1

Overview of class I ribonucleotide reductases

Key features	Class Ia	Class Ib	Class Ic ^a
α^b	NrdA	NrdE	NrdA
β^c	NrdB	NrdF	NrdB
Active RNR	$\alpha_2 \beta_2?$ (<i>E. coli</i>) ^d $\alpha_6 \beta_2?$ $\alpha_6 \beta_6?$ (human)	$\alpha_2 \beta_2?$	$\alpha_2 \beta_2?$
SA ^e	6,000–8,000 (<i>E. coli</i>) 3,000–4,000 (human)	See Table 2	600
Metallocofactor			
In vitro	Fe ^{III} Fe ^{III} -Y•	Mn ^{III} Mn ^{III} -Y• Fe ^{III} Fe ^{III} -Y•	Mn ^{IV} Fe ^{III} Fe ^{IV} Fe ^{III} ^f
In vivo	Fe ^{III} Fe ^{III} -Y•	Mn ^{III} Mn ^{III} -Y• Fe ^{III} Fe ^{III} -Y• ? ^g	Mn ^{IV} Fe ^{III} ?
Y•/ β_2	1.2 (<i>E. coli</i>) 1.0 (human)	See Table 2	1.5 Mn ^{IV} Fe ^{III} / β_2
Accessory factors			
Reductant	Thioredoxin Glutaredoxin	NrdH	Unknown
Cofactor assembly	YfaE (<i>E. coli</i>)	NrdI	Unknown

^a *C. trachomatis*.^b Subunit in which nucleotide reduction occurs.^c Subunit that harbors the dinuclear metal cluster.^d ?, not known for certain at present.^e Specific activity, nmol CDP produced min⁻¹ (mg β_2)⁻¹.^f Probably inactive.^g There is no evidence to suggest a Fe^{III}Fe^{III}-Y• cofactor is physiologically relevant in any class Ib RNR, but the possibility cannot be excluded at present.

Table 2
Class Ib ribonucleotide reductase properties: Y• content, metal loading, and activity

Source	Mn ^{III} Mn ^{III} - Y• cofactor		Fe ^{III} Fe ^{III} - Y• cofactor		References
	Y•/β2	Mn/β2	SA ^a	Y•/β2	
<i>E. coli</i>					
As isolated ^b	0.2	0.9	720	–	33
Reconstituted	0.25	1.4	600	3.6–3.8	31, 107
<i>C. ammoniagenes</i>					
As isolated	0.36 ^c	1.5 ^c	69,000 ^c	1.0 ^d	32 ^c , 111 ^d
Reconstituted	–	–	–	3.0	111
<i>B. subtilis</i> ^e					
As isolated	0.4–0.5	1.8–2.4	70–160	0.9	5
Reconstituted	0.6	1.8–2.2	73	2.6	9
<i>S. Typhimurium</i> ^f					
As isolated	–	–	–	3.6	830
Reconstituted	–	–	–	3.2	325
<i>M. tuberculosis</i> ^f					
As isolated	–	–	–	0.3–0.4	120
<i>B. anthracis</i> ^f					
Reconstituted	–	–	–	0.4–0.5	3
<i>S. pyogenes</i> ^f					
As isolated	–	–	–	1.0	169
As isolated	–	–	–	2.4	101

^a nmol dCDP produced min^{−1} (mg β2)^{−1}.

^b Purified from endogenous levels.

^c Overexpressed in *C. ammoniagenes*.

^d Overexpressed in *E. coli* in rich medium.

^e Y. Zhang & J. Stubbe, in preparation. Protein containing Mn^{III} Mn^{III} - Y• cofactor was isolated by overexpression of the entire class Ib operon in *B. subtilis*. Protein containing Fe^{III} Fe^{III} - Y• cofactor was isolated by overexpression of NrdF in *E. coli*.

f_j Overexpressed in *E. coli*. See table S1 in Reference 107 for references.

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