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

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Abstract

Ribonucleotide reductases (RNRs) convert nucleotides to deoxynucleotides in all organisms. Activity of the class Ia and Ib RNRs requires a stable tyrosyl radical (Y•), which can be generated by reaction of O₂ with a diferrous cluster on the β subunit to form active diferric-Y• cofactor. Recent experiments have demonstrated, however, that in vivo the class Ib RNR contains an active dimanganese(III)-Y• cofactor. The similar metal binding sites of the class Ia and Ib RNRs, their ability to bind both Mn^{II} and Fe^{II}, and the activity of the class Ib RNR with both diferric-Y• and dimanganese(III)-Y• cofactors raises the intriguing question of how the cell prevents mismetallation of these essential enzymes. The presence of the class Ib RNR in numerous pathogenic bacteria also highlights the importance of manganese for these organisms' growth and virulence.

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

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Diferric-Y• assembly in class Ia RNRs

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

The diferric-Y• cofactor can self-assemble in vitro [13] from apo- β , Fe^{II}, O₂, and reductant by an extensively studied mechanism (Figure 2a) [14–17]. The same basic mechanism is expected to operate in vivo, but the requirements for Fe^{II} loading and the donor of the essential reducing equivalent led our laboratory to investigate how these steps occur inside the cell.

The observation that functionally related genes are often organized within operons has provided the first insight into the machinery involved in the biosynthesis and maintenance of the class Ia and Ib RNR cofactors. Bioinformatic analyses of genomes encoding class Ia RNRs revealed that 29% of the operons containing the genes for the two class Ia RNR subunits (*nrdA* and *nrdB*, *E. coli* nomenclature) also contained a gene that codes for a [2Fe2S]-ferredoxin, *yfaE*. While this gene is not essential, an *E. coli* $\Delta yfaE$ strain grown in minimal media in the presence of hydroxyurea, a scavenger of the Y•, exhibited a growth rate one-third that of the isogenic wild-type strain. In vitro experiments demonstrated that YfaE is able to rapidly reduce the Y•-reduced (met) diferric cluster to a diferrous cluster, which rapidly self-assembles into active cluster upon addition of O₂ [18] (Figure 2a). Further in vitro studies have also suggested that YfaE is competent to deliver the extra reducing equivalent required for cluster assembly [18]. Whole cell EPR and western blotting analyses of *E. coli* in which β manipulated from 4 μ M to 3.3 mM have demonstrated that Y• levels can be modulated in vivo, supporting the physiological relevance of the proposed maintenance pathway [19].

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

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

Class Ia and Ib RNRs are structurally homologous but contain different metal cofactors

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

The self-assembly of active diferric-Y• cofactors in class Ib RNRs and the presence of identical metal ligands in the class Ia and Ib RNRs suggested to many researchers that the class Ib RNR contained a diferric-Y• inside the cell [31]. However, studies of *Corynebacterium ammoniagenes* [32] and *Bacillus subtilis* [33] have demonstrated the essentiality of Mn for growth. Early biochemical studies of *C. ammoniagenes* NrdF suggested the presence of Mn in the active cofactor [34,35], but Y• was not detected and RNR activity was very low. Furthermore, attempts to generate active NrdF in vitro using Mn^{II} and a variety of oxidants, including O₂ and H₂O₂, were unsuccessful [31]. Our recent experiments elucidating the role of NrdI in the *E. coli* class Ib system and studies of Auling and coworkers purifying *C. ammoniagenes* NrdF from its native organism have both established the activity of a dimanganese(III)-Y• (Mn^{III}₂-Y•) cofactor.

Dimanganese(III)-Y• cofactor formation in vitro

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

Several in vitro observations supported this proposal. First, affinity chromatography of Mn^{II}₂NrdF and tagged NrdI indicated a relatively tight interaction [5]. Second, when Mn^{II}₂-NrdF was incubated with the fully reduced form of NrdI (NrdI_{hq}) in an anaerobic chamber and then exposed to O₂, a dimanganese(III)-Y• cofactor was generated which was active in nucleotide reduction [5]. These and other biochemical studies suggested that the role of NrdI_{hq} in cofactor assembly is to reduce O₂ to either H₂O₂ or HO₂⁻ [HOO(H)], which is channeled directly to the metal site through a NrdI•NrdF complex.

Support for this oxidant channeling model is provided by recent structures of Mn^{II}₂-NrdF alone and in complex with oxidized NrdI and NrdI_{hq}, as well as of Fe^{II}₂-NrdF [39]. These structures have also provided insight about the role of the carboxylate ligands of the metal

clusters in providing O₂ access to the Fe^{II}₂ cluster and HOO(H) access to the Mn^{II}₂ cluster. The Mn^{II}₂ cluster of NrdF has a distinct coordination mode of Glu158, never before observed in any RNR βs or related diiron-carboxylate proteins (compare diferrous and dimanganese(II) forms, Figure 1b), and a unique water coordinated to Mn2. The different positions of Glu158 in Fe^{II}₂- and Mn^{II}₂-NrdF result in different routes for the O₂ and HOO(H) oxidants to access the respective metal sites. In Fe^{II}₂-NrdF, the channel terminates at a hydrophobic pocket trans to His195, while in Mn^{II}₂-NrdF, it ends at the water bound to Mn2 [39] (Figure 3). HOO(H) is therefore proposed to replace the water at Mn2 in the first step of cluster assembly [39]. Thus, the structures offer clues as to the distinct mechanisms of Fe^{III}₂-Y• and Mn^{III}₂-Y• cofactor generation and the role of NrdF in catalyzing both processes with different oxidants.

Dimanganese(III)-Y• cofactor formation in vivo

Nearly three decades of effort have recently culminated in the successful isolation of highly active NrdF from *C. ammoniagenes* by the Auling laboratory [6]. This isolation required the overexpression of NrdF in *C. ammoniagenes* to 5% of total cellular protein and growth in the presence of 185 μM Mn^{II}. The purified protein contained 1.5 Mn/β2 and 0.36 Y•/β2 [6]. Extensive characterization of this protein by EPR spectroscopy revealed a Mn^{III}₂-Y• cofactor with a spectrum very similar to the reconstituted Mn^{III}₂-Y• cofactor in *E. coli* NrdF. A high-resolution crystal structure of the *C. ammoniagenes* NrdF, proposed to be in the Mn^{III}₂ state, has also been reported [6] and shows that Glu158 has undergone a dramatic carboxylate shift in the course of cofactor assembly relative to the *E. coli* Mn^{II}₂ structure (Figure 1b). These structures support the importance of ligand reorganization, first suggested by the Lippard group, in reactions of dinuclear redox centers [40].

Very recent studies in *E. coli* and *B. subtilis* complement the studies in *C. ammoniagenes*. *E. coli* NrdF has been purified from *E. coli* GR536, a strain deficient in all known iron uptake systems and requiring Mn for normal growth [41], using a NrdI affinity column and additional columns. The protein was purified 3000-fold to homogeneity [7] and contained 0.9 Mn and 0.2 Y•/β2, comparable to the levels observed in the in vitro reconstitution experiments and those in *C. ammoniagenes* NrdF. *B. subtilis* NrdF has also recently been purified after upregulation of the entire class Ib RNR operon and shown to contain a Mn^{III}₂-Y• cofactor [8]. In all three cases, Mn loading and Y•/β2 is substoichiometric, perhaps related to the genetic manipulation and growth conditions used to observe the cofactor. These studies together suggest that many class Ib RNRs will use Mn^{III}₂-Y• cofactors. However, the possibility that some organisms use Fe^{III}₂-Y• cofactors cannot be excluded at present and must be evaluated by purification of these proteins from the native organisms, a challenge given the low concentrations of RNR and potential Y• instability.

Mechanism of Mn^{III}₂-Y• cofactor assembly

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

Conclusion: Control of metallation in vivo

The metal binding sites of class Ia and Ib β s are identical and both are active with diferric- Y^\bullet cofactors, but class Ib RNRs use a Mn^{III}_2 - Y^\bullet cofactor in vivo. Mn^{II} binds more tightly than Fe^{II} to *E. coli* NrdB [44], but the Mn^{II}_2 form cannot be converted to an active form containing Y^\bullet . These observations emphasize the importance of understanding how the cell ensures correct metallation of metalloenzymes such as the class I RNRs¹.

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

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

Research Highlights

- Definitive evidence for a Mn_2 cluster in class Ib RNRs has been recently provided
- NrdI is essential in supplying the oxidant for class Ib dimanganese cluster assembly
- Class Ib RNRs are active with Fe or Mn; control of metal loading is critical in vivo
- Class Ib RNR structures reveal carboxylate shifts during metal center oxidation

References

**Of outstanding interest

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

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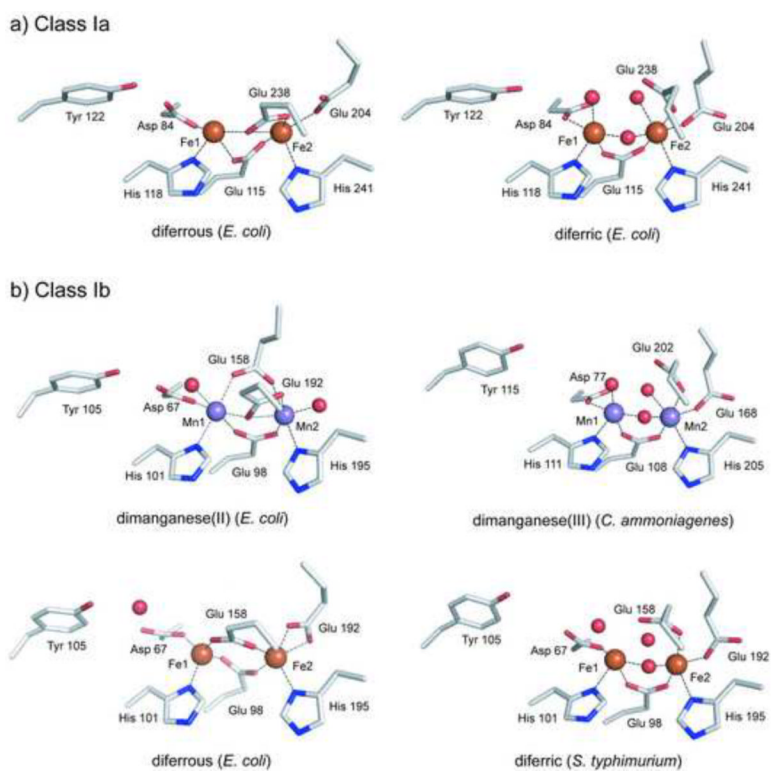


Figure 1. Structures of the reduced and oxidized cofactors of the class Ia and Ib RNRs. Iron and manganese ions are depicted as brown and purple spheres, and solvent molecules are shown as red spheres. Generated using PyMOL from PDB files 1PIY (diferrous NrdB), 1MXR (diferric NrdB), 3N37 [dimanganese(II) NrdF], 3MJ0 [dimanganese(III) NrdF], 3N38 (diferrous NrdF), and 2R2F (diferric NrdF).

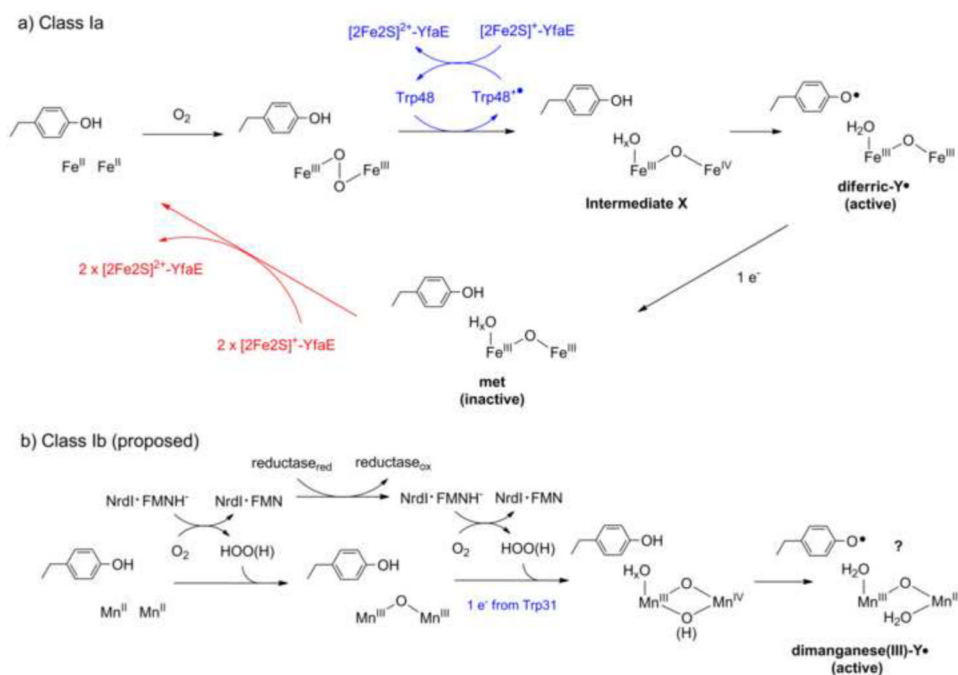


Figure 2. Mechanisms of cofactor assembly (black) and maintenance (red) for class Ia and Ib RNRs. The intermediates shown in the class Ia mechanism have been spectroscopically characterized, whereas those in the class Ib mechanism are postulated based on analogy to the class Ia pathway. The structure of the dimanganese(III)-Y• cofactor shown is based on the crystal structure of *C. ammoniagenes* Mn^{III}₂-NrdF [6], but the possibility of photoreduction during data collection makes the identity of the bridging ligands in the active cofactor unclear. Blue text indicates the involvement of the “extra” electron in cofactor assembly.

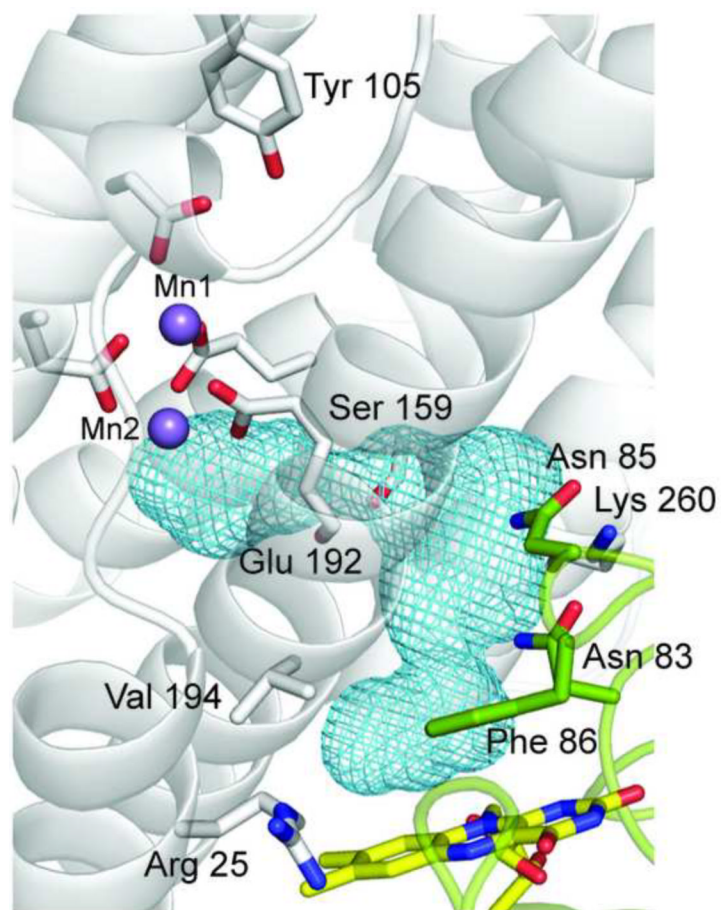


Figure 3. The oxidant channel in the $\text{Mn}^{\text{II}}_2\text{-NrdF}\cdot\text{NrdI}_{\text{ox}}$ complex. Binding of NrdI (green) to NrdF (gray) extends a hydrophilic channel (blue mesh) between the metal site and the flavin cofactor in NrdI (yellow sticks). Residues shown in sticks are highly or completely conserved in NrdIs and NrdFs. Reproduced from ref. [39].