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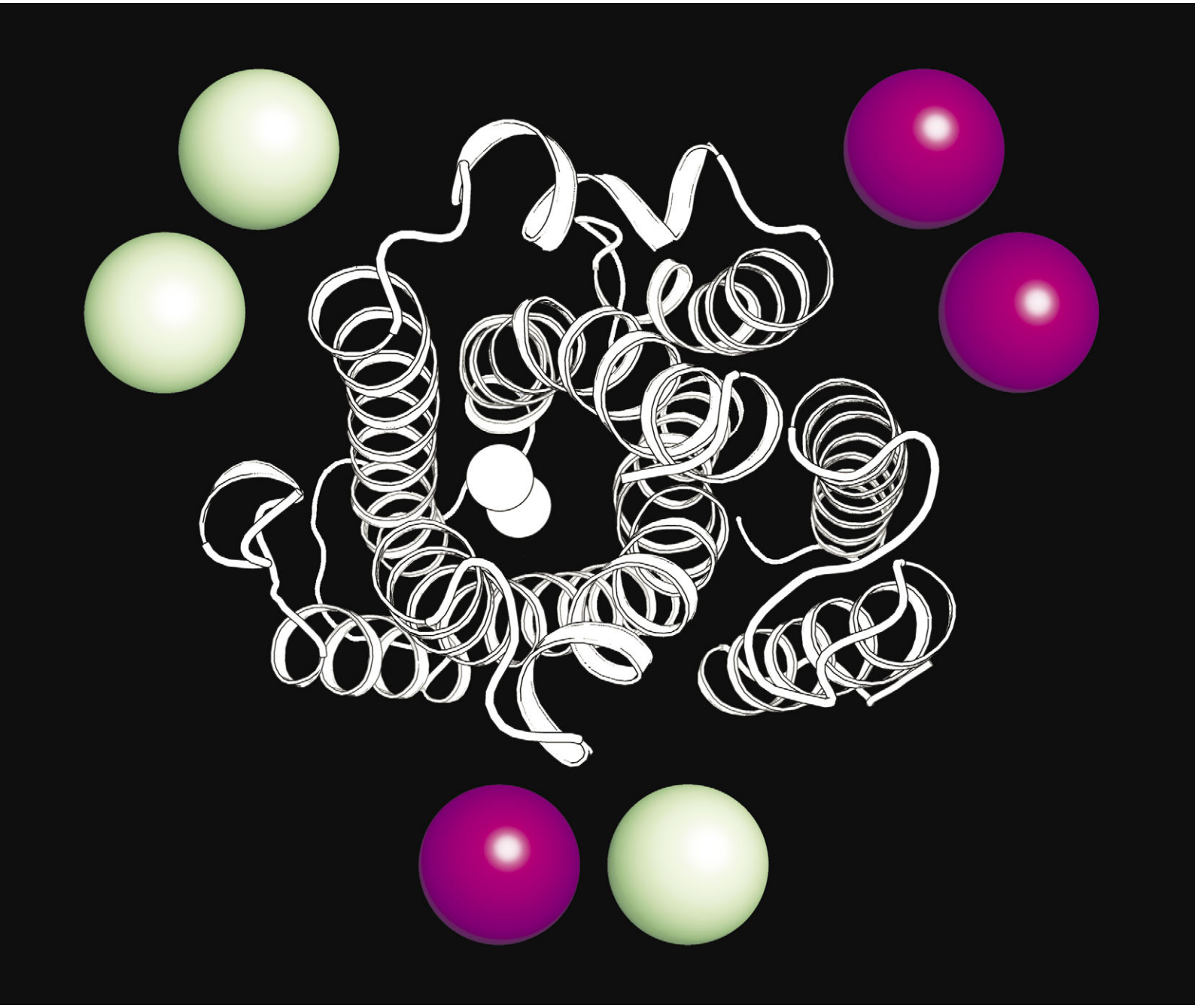

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CRITICAL REVIEW

Metal use in ribonucleotide reductase R2, di-iron, di-manganese and heterodinuclear—an intricate bioinorganic workaround to use different metals for the same reaction

Martin Högbom

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The ferritin-like superfamily comprises of several protein groups that utilize dinuclear metal sites for various functions, from iron storage to challenging oxidations of substrates. Ribonucleotide reductase R2 proteins use the metal site for the generation of a free radical required for the reduction of ribonucleotides to deoxyriboinucleotides, the building blocks of DNA. This ubiquitous and essential reaction has been studied for over four decades and the R2 proteins were, until recently, generally believed to employ the same cofactor and mechanism for radical generation. In this reaction, a stable tyrosyl radical is produced following activation and cleavage of molecular oxygen at a dinuclear iron site in the protein. Discoveries in the last few years have now firmly established that the radical generating reaction is not conserved among the R2 proteins but that different subgroups, that are structurally very similar, instead employ di-manganese or heterodinuclear Mn–Fe cofactors as radical generators. This is remarkable considering that the protein must exercise a strict control over oxygen activation, reactive metal–oxygen intermediate species and the resulting redox potential of the produced radical equivalent. Given the differences in redox properties between Mn and Fe, use of a different metal for this reaction requires associated adaptations of the R2 protein scaffold and the activation mechanism. Further analysis of the differences in protein sequence between R2 subgroups have also led to the discovery of new groups of R2-like proteins with completely different functions, expanding the chemical repertoire of the ferritin-like superfamily. This review describes the discoveries leading up to the identification of the different Mn-containing R2 protein groups and our current understanding of them. Hypotheses regarding the biochemical rationale to develop these chemically complex alternative solutions are also discussed.

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Introduction

What is a native metal cofactor?

When discussing with colleagues there seems to be a consensus that the term "native" metal cofactor denotes the element that provides the main contribution to the primary function of a given protein *in vivo*. Though this appears simple and obvious, in practice it can be very hard to define, at least for the vast majority of proteins that are naturally present in such a low abundance that the protein cannot be isolated in biochemical quantities from the native source at natural expression levels.

Metal cofactor assignment is usually done by analyzing the metal content of a protein overexpressed in a non-natural environment while not taking into account the metal status of the cell, *e.g.* initial metal availability or how overexpression of a metalloprotein may titrate out a particular metal. The environment of the cell also controls metal incorporation, both specifically and generally, by the influence of metal chaperones and competing proteins.[1,2] Cofactor assignment

is also commonly done by studying how the addition of a certain metal influences a particular catalytic reaction. This basis for the assignment is of course valid at the chemical level for the isolated system under study. However, it is not necessarily true that the native cofactor (as defined above) provides the highest specific activity for a certain reaction under given experimental conditions. The powerful spectroscopic and crystallographic methods presently available for studying metalloproteins generates very detailed information on the metal site, and their importance is undisputed. Still, most techniques rely on obtaining large amounts of a highly purified sample and are thus intrinsically biased by assumptions on what components make up the complete functional system. As we will see in this review, what is regarded as the native cofactor can change entirely, even for systems that have been thoroughly studied for decades.

The ferritin-like superfamily

The ferritin-like superfamily of proteins, sometimes also called iron-oxygen proteins, share a metal-binding core 4-helix bundle with a distinctive topology, including a crossover connection between helices 2 and 3 (Fig. 1).[3–6] They form a histidine and carboxylate-coordinated di-metal site in the center of the structurally conserved 4-helix bundle (Fig. 2). The metal site binds two metal ions in the 2+ oxidation state that are subsequently oxidized either by molecular oxygen or another oxygen-containing oxidant, *e.g.* a peroxide species. The resulting oxidized metal site can then be used for various functions, described briefly below.[3–9]

The superfamily includes proteins of several different functions and levels of structural and chemical complexity. The Iron-storage proteins ferritins and bacterioferritins form 24-meric assemblies used in all domains of life for iron storage, up to 5000 iron atoms can be stored inside the hollow shell as a Fe(III)-oxo-hydroxide mineral core.[10,11] DPS proteins (DNA-binding proteins from starved cells) are another group, structurally very similar to ferritins, and are also involved in iron homeostasis but form dodecameric hollow complexes instead. In addition, they appear involved in DNA binding and protection against radical damage. DPS proteins differ from the other proteins in the ferritin-like superfamily by not forming the same type of di-metal site in the 4-helix bundle but instead bind metals between protomers in the dodecameric complex.[11–14]

When the reduced metal site reacts with molecular oxygen a number of potentially very reactive metal–oxygen intermediates can be produced. The ferritin superfamily also contains a number of groups of proteins that utilize this reactive inorganic core for challenging substrate oxidations. For example the acyl-ACP desaturases, diiron proteins that insert a double bond in an acyl chain bound to acyl carrier protein (ACP).[15–18] Arguably, the two best-studied groups of the ferritin-like superfamily are the ribonucleotide reductase R2 proteins and the bacterial multicomponent monooxygenases (BMMs), to which the di-iron methane monooxygenase (MMO) belongs. BMMs use the diiron cofactor to perform extremely chemically demanding two-electron oxidations. For example, MMO generates an oxo-bridged Fe(IV)–Fe(IV) intermediate which hydroxylates methane to methanol, thus activating the strongest C–H bond of any hydrocarbon.[19–21] The family also includes various other hydrocarbon oxidases capable of oxidizing C1–C8 alkanes and alkenes as well as aromatic substrates. These enzymes have different and usually broad substrate specificities, for this reason the proteins and the bacteria that carry them are of great interest for industrial and environmental applications such as bioremediation of contaminated soil. BMMs are multi-subunit complexes requiring different protein components for activity.[22–26]

Ribonucleotide reductases (RNRs) are the only identified enzyme systems for *de novo* synthesis of deoxyribonucleotides. The R2 protein subunit of Class I RNR (Fig. 3) belongs to the ferritin-like superfamily and generates a radical essential for catalysis. Chemically, the R2s thus differ from the BMM group in that they perform a one-electron oxidation (radical generation) as opposed to the two-electron oxidations performed by the BMMs. Much effort has gone into defining the structural and chemical determinants that direct the system to perform one- rather than two-electron chemistry.[23,27–29] Another difference is that the standard R2 proteins in a sense carry their own substrate, a tyrosine residue that becomes oxidized to a tyrosyl radical. For this reason they do not contain a substrate-binding active site like the BMMs do. Unlike the other proteins of the Ferritin-like superfamily, R2 proteins display one metal coordinating aspartate residue (Fig. 2, position A) and three glutamate residues as opposed to four glutamate residues. The reaction and metal requirement for this group of proteins is the focus of this review and will be described in more detail below.

There is only one group of proteins in the ferritin superfamily that were initially characterized as utilizing a manganese (*cf.* Iron) cofactor. The manganese catalases display a very similar dinuclear metal site albeit lacking one of the bridging carboxylates. Manganese catalases catalyze the disproportionation of hydrogen peroxide to water and molecular oxygen involving redox changes at the metal site.[7–9]

The ferritin-like superfamily has attracted significant interest because of its fascinating bioinorganic chemistry of oxygen activation and intricate control of high-valent metal cores and electron transfer. There is also a great industrial interest in these systems that perform selective and non-complete oxidations of organic substances using molecular oxygen as an oxidant; reactions that are still largely outstanding goals for chemical industry. Potential industrial uses include both the native chemistry of, *e.g.* BMM proteins for synthesis and bioremediation purposes[22–26] or modified, primarily ferritin proteins, as scaffolding templates for fabrication of different structures, encapsulation of nanoparticles, or scaffolds for targeted delivery of various substances.[30]

Ribonucleotide reductases

As mentioned above, ribonucleotide reductases are the only identified enzyme systems for *de novo* synthesis of all four deoxyribonucleotides, the building blocks of DNA. The enzyme performs reduction of all four ribonucleotides to their corresponding deoxyribonucleotides while it also, controlled by allosteric regulation, maintains balanced pools of the deoxyribonucleotides. RNR is also a drug target for antiproliferative

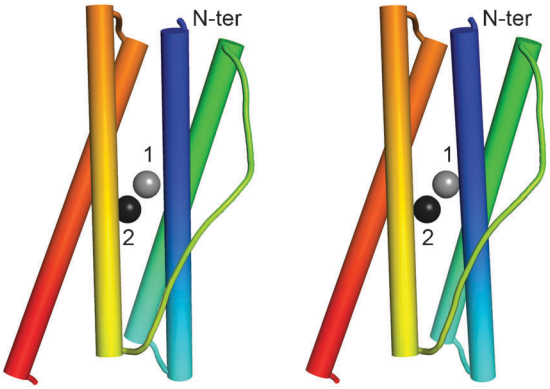

Fig. 1 Stereo figure showing the general topology of the core 4-helix bundle of the ferritin-like superfamily. The cross-over connection between helices 2 and 3 provides the distinctive topology of the metal-coordinating bundle. Metal positions 1 and 2 are indicated in gray and black respectively.

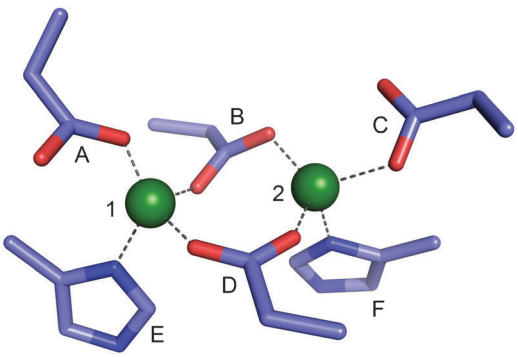

Fig. 2 Architecture of the metal coordinating residues in ferritin-like proteins. This structure depicts the *E. coli* Class-Ia R2 protein in its reduced (Fe(II)–Fe(II)) form. The exact coordination geometry differs between proteins and the oxidation state of the metal site. Bacterioferritins, R2 proteins, acyl-ACP desaturases and the bacterial multicomponent monooxygenases possess, as shown in the figure, two terminal carboxylate ligands, one bridging carboxylate and one carboxylate (position D) that can assume bridging or terminal positions depending on the oxidation state of the metal site. The standard R2 proteins differ from the other groups in that position A is an Aspartate residue as opposed to Glutamate. Ferritins lack one Histidine ligand (position F) and display a Glutamine in position D and also an additional carboxylate. Manganese catalases lack the carboxylate in position D.

drugs for treatment of *e.g.* viral infections and cancer. Three main classes of ribonucleotide reductase have been identified.[31–39] Class I is dependent on oxygen for activity, Class II can function both in aerobic and anaerobic environments, while Class III is very oxygen sensitive and activity is lost upon exposure to oxygen. Structures of the catalytic (R1) subunits from all different classes have been solved,[40–42] and show great structural similarity. All three classes have a conserved cysteine residue at the active site located at the tip of a loop in the centre of an unusual 10-stranded α/β-barrel. All classes are believed to have evolved from a common ancestor and utilize the same cysteine thiyl radical mechanism for ribonucleotide reduction. As the reaction is a net two-electron process, the radical is recovered at the end of the reaction.[43–47]

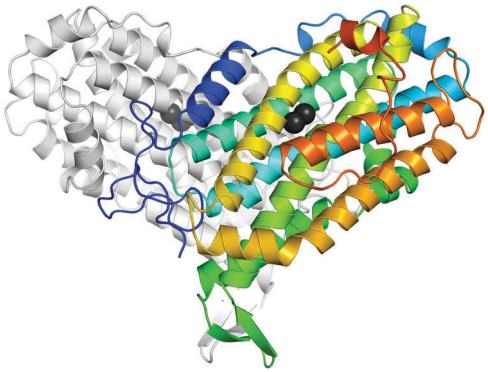

Fig. 3 Structure of the *E. coli* Class Ia R2 protein homodimer. One monomer is colored from blue (N-terminus) to red (C-terminus); the second monomer is in light gray. Iron ions are shown as black spheres.

The catalytic mechanism is thought to be conserved among the groups and the major difference between the classes is the way that the active site cysteinyl radical is generated. In Class II, the radical is generated by homolytic cleavage of the carbon–cobalt bond in an adenosylcobalamin cofactor directly bound to the catalytic subunit.[40,48–50] Classes I and III, on the other hand, utilize separate proteins that work as radical generators. The activase of Class-III ribonucleotide reductase belongs to the radical-SAM family of enzymes where the radical is initially generated by homolytic cleavage of S-adenosyl-methionine (SAM) at an iron–sulfur center.[51–54]

Class I ribonucleotide reductase and the R2 protein

The oxygen-dependent Class-I RNRs are present in eukaryotes, bacteria, bacteriophages and viruses and are composed of two different homodimeric proteins, R1 containing the active site, and R2 that generates, stores and delivers the radical essential for activity. The radical transfer from R2 to R1 is shown to proceed along an array of invariant hydrogen bonded residues leading from the diiron site in R2 to the active site in R1. After substrate reduction, the radical is delivered back to the R2 subunit and stored for use in the next turnover.[33,36,45–47,55–57] Class I is further divided into subclasses Ia, Ib and recently, Ic. The basis of this division is somewhat incoherent and based on sequence similarity, operon arrangement, properties of allosteric regulation, and properties of the radical cofactor. The R2 proteins from the different classes are commonly denoted R2 (Class Ia), R2F (Class Ib), and R2c (Class Ic).[33,39,58,59]

Class Ia is the most extensively studied of these three, mainly because of the vast amount of knowledge acquired for the *E. coli* Class Ia RNR. This protein will serve as the foundation for our description of the fascinating story regarding the mechanism and metal requirements of R2 proteins. This story has developed during more than four decades and, as soon as the field appeared settled, has continually presented us with surprises that require reconsideration of what we had accepted as true. The last few years have been particularly revealing regarding the metal requirement for these proteins, which is the focus of this review.

The *E. coli* R2 protein was shown to contain iron in the late 1960's[60] and was the first protein in which a protein radical

was observed.[61] The radical was later identified to reside on a tyrosine residue by isotopic labelling[62] and finally assigned to Tyrosine 122 by mutational studies.[63] Since these seminal discoveries, *E. coli* RNR R2 has been used as a model system for a great number of studies directed at understanding the chemistry of ribonucleotide reduction, diiron-catalyzed oxygen activation, long range electron transfer, high-valent iron-oxygen chemistry, and radical generation and stabilization in proteins. Up until a few years ago, it was generally believed that all R2 proteins functioned and performed radical generation and storage analogous to the *E. coli* Class-Ia R2.

The overall reaction can be formally written as follows:

$$\text{R2-Y122-OH} + \text{(Fe(II) Fe(II)} + \text{O}_2 + \text{H}^+ + \text{e}^- \rightarrow$$
$$\text{R2-Y122-O}^\bullet + \text{(Fe(III)-O}^{2-}\text{-Fe(III))} + \text{H}_2\text{O}$$

The four-electron reduction of dioxygen is accomplished by two electrons from the iron ions, one from an external reductant, and one from Tyrosine 122. The external electron is believed to be injected *via* a hydrogen bonded radical transfer pathway leading from the diiron site to the surface of the protein. This is part of the same path by which the radical is transferred to the R1 subunit when the substrate reduction is to take place.[33,36,39,45–47,59,61,62,64–71]

There are a number of states of the R2 protein, as well as reaction intermediates, that have been structurally and spectroscopically characterized. There are crystal structures of the folded but metal-free apo protein[72] and the reduced form, resulting from the binding of two ferrous iron ions to the apo protein.[73] The reduced form of the protein then reacts with molecular oxygen to produce the tyrosyl radical. In the wild-type protein, a key intermediate 'X', directly preceding the tyrosyl radical, has been isolated and extensively characterized.[29,71,74–77] At this stage of the reaction, the oxygen atoms are fully reduced and the external electron has entered the site, X is described as an oxo-bridged, formally Fe(III)–Fe(IV), site with a coordinated hydroxide. Intermediate X then decays to form the active state with a diferric μ-oxo-bridged site and the tyrosyl radical.[61] The active state has also been structurally characterized by a combination of X-ray and EPR crystallography.[78] Reduction of the radical renders the protein in the resting "met" state, with a μ-oxo-bridged diferric site without the radical.[6] In the mutant proteins D84E and D84E/W48F, an intermediate 'P', preceding intermediate X has also been observed and assigned as a symmetric μ-1,2 peroxo diferric species.[27,79,80]

Metal requirement and binding in R2 proteins

The complex set of reactions performed by R2 proteins, including oxygen activation, managing highly reactive metal–oxygen species, tuning of redox potentials and the control of radical transfer would be expected to be stringently kept in terms of the chemistry, the cofactor and the protein, which acts as both the functional scaffold and as an insulator, protecting the surrounding cellular components from the highly reactive inorganic core. Indeed, the several hundred studies of class-I RNR and R2 proteins from different species and both subclasses Ia and Ib appeared to agree with the general scheme above. *In vitro* activity showed a strict requirement for iron and the radical harboring tyrosine, that was completely conserved among the many hundred sequenced genes encoding R2 proteins.[33–36,39,45–47,59,62,81] Because manganese and iron have similar primary ligand preferences it is not surprising that manganese can be incorporated in apo R2 proteins when subjected to Mn(II) *in vitro*. This was also done in a number of studies where Mn served as a spectroscopic probe or was otherwise used to study metal binding and the conformational space available to the metal ligands at different oxidation states.[82–85] However, no RNR activity was observed for these Mn-containing proteins.

Class Ic R2 proteins

The background to the third subclass, Ic, of R2 proteins started in the early 1990's with the identification of ribonucleotide reductase activity in the intracellular parasite and human pathogen *Chlamydia trachomatis*.[86] This finding is consistent with the observation that *C. trachomatis* cannot acquire deoxyribonucleotides from the host and it thus needs to be synthesized by the parasite.[87] The *C. trachomatis* RNR genes were cloned in 2000 and the overexpressed proteins showed RNR activity *in vitro*. The gene sequences placed them among the Class-Ia RNRs but there was, however, one peculiar feature.[88] Direct alignments of the R2 protein sequence showed that it contained a phenylalanine where the otherwise completely conserved and essential radical harboring tyrosine was usually located. A tyrosine residue was, however, positioned two residues downstream in the sequence and was hypothesized to assume the radical harboring role. Mutation of this tyrosine to a phenylalanine yielded an inactive protein, providing support to the hypothesis.[88] A more subtle difference was that the terminal carboxylate ligand to metal position 1 (Fig. 2, position A) was a glutamate, unlike other R2 proteins that all have an aspartate residue in this position.

The structure of the *C. trachomatis* protein was solved in 2004.[58,89] The structure showed that a phenylalanine indeed occupied the otherwise radical harboring position. The downstream tyrosine, previously suggested to be the radical position, was exposed to solvent and thus a very unlikely radical harboring site. Its importance for activity was instead more likely related to the interaction with the R1 subunit. The *C. trachomatis* R2 protein, overexpressed in *E. coli*, contained close to two equivalents of iron per polypeptide. Production of the metal-depleted protein allowed reconstitution with ^{56}Fe(II) and ^{57}Fe(II). Upon reaction with molecular oxygen this yielded a relatively stable high-valent Fe(III)–Fe(IV) metal site, similar to the key intermediate, X, in standard R2 proteins. It was suggested that this radical equivalent oxidation state of the metal site replaced the tyrosyl radical in standard R2 proteins.[58] Interestingly, database searches identified a number of sequences from other organisms that also possessed these particular features and were therefore assigned to a new subgroup of tyrosyl radical-less R2 proteins, Class Ic.[58] It was further hypothesized that the lack of the tyrosyl radical could be an adaptation to produce a system more resistant to certain radical scavengers *e.g.* produced by the immune response of the host. Further studies showed that the stability of the Fe(III)–Fe(IV) state was greatly enhanced in the presence

of protein R1 and the substrate[90] and that the amount of this species was linked to the catalytic activity,[91] lending further support to the hypothesis that the Fe(III)–Fe(IV) species is involved in reaction initiation. The Fe(III)–Fe(IV) state was also characterized by combined EPR and ENDOR spectroscopies and shown to be practically identical to the intermediate X in the *E. coli* Class Ia protein.[92]

There was still a surprise waiting in this system. In 2007 Jiang and coworkers noted that the activity of the *C. trachomatis* R2 protein from different preparations did not appear to correlate with iron content. Reconstitution of the metal-depleted protein showed that the highest activity was obtained with a 1 : 1 ratio of Fe(II) to Mn(II) suggesting a heterodinuclear cofactor in the protein. EPR and Mössbauer spectroscopy combined with oxidation/reduction experiments identified the active species as a Mn(IV)–Fe(III) oxidation state of the heterodinuclear cofactor, formed upon reaction of the Mn(II)–Fe(II) site with O_2.[93] The higher specific activity with a mixed Mn–Fe cofactor was also independently discovered and confirmed by Voevodskaya and coworkers,[94] published three weeks after the Jiang study.

This discovery was rapidly followed by a number of important biochemical, spectroscopic and computational studies describing the formation and properties of the novel heterodinuclear cofactor and the radical transfer and inhibition properties of the *C. trachomatis* R2.[95,96–101] For recent reviews see Ref. 102–105. Notably, it was shown that the reconstitution reaction proceeds *via* a Mn(IV)–Fe(IV) intermediate state.[106] This is interesting because the Fe(IV)–Fe(IV) intermediate has not been observed in standard R2 proteins although it has been observed in 2-electron oxidases such as MMO.[19] Interestingly it was also shown that, unlike the diiron systems, not only was the activity of the *C. trachomatis* Mn–Fe protein stable against incubation with H_2O_2 but the reduced forms of the protein were quantitatively oxidized to yield the active form of the protein.[107] Thus showing that this protein indeed has an altered reactivity profile against potential radical scavengers.

There is now no doubt that the *C. trachomatis* R2 protein shows highest activity with a heterodinuclear Mn–Fe site, however, there are still conflicting views whether the diiron enzyme is completely inactive, or if it shows significantly lower activity. This may seem like a moderately important question but it has great relevance for our understanding of the bioinorganic chemistry of the R2 proteins and the intricate process of reversible radical transfer to the R1 subunit. In general, the views regarding this point appear to be divided according to continent, the results from the European groups suggest a low activity with a diiron cofactor, while the US groups propose that the residual activity is due to contaminating manganese in the preparations. Based on available published data, this question is difficult to conclusively resolve. This issue of course relates to the fundamental question of *why* a heterodinuclear site is needed when no tyrosyl radical is generated. Using a computational approach Roos and Siegbahn propose an elegant rationale.[108] Their calculations suggest that the Mn(IV)–Fe(III) redox state in the *C. trachomatis* R2 protein is an equally strong oxidant as the (Fe(III)–Fe(III)–Y$^{\bullet}$) radical site in *E. coli* R2 and is thus balanced with the cysteinyl radical active site in the R1 subunit. This would, as in standard R2 proteins, allow reversible radical transfer and numerous turnovers in the R1 subunit without the need to regenerate the radical at the metal site in R2. The Fe(IV)–Fe(III) species, however, would be too strong an oxidant to allow reversible radical transfer.[108] This explanation, in principle, allows for a much less efficient single-turnover reaction where the radical has to be regenerated in the diiron R2 protein by the reduction and subsequent re-oxidation of the metal site for every turnover of the R1 protein.

Metal requirement of Class Ib revisited

In the 1980's there were a couple of papers describing an apparent outlier RNR. Early studies established that growth and DNA synthesis in *Corynebacterium* (prev. *Brevibacterium*) *ammoniagenes* are impaired during manganese starvation.[109] This was speculated to be due to a lack of ribonucleotide reductase activity. Experiments done *in vivo* or with whole-cell extracts showed that RNR activity from Mn depleted cells could be recovered by addition of Mn(II), the addition of Fe(II) also restored activity although only to a level of about half of what was achieved using Mn(II).[110] The purified enzyme was shown to be active and contain manganese, but after metal removal activity could not be restored by addition of metal ions.[111] Based on these observations, the *C. ammoniagenes* R2 was assigned as manganese dependent by Auling and coworkers. Later, a radical was observed in the protein[112] and the metal site suggested to be mono Mn by EPR and metal stoichiometry measurements.[113] Based on these results, the *C. ammoniagenes* R2 was suggested to belong to a new class (IV) of ribonucleotide reductases.[34,114] Sequencing of the gene, however, later placed the protein among the Class-Ib RNRs.[115] In subsequent experiments, the heterologously overexpressed and purified protein showed no radical generation or activity when reconstituted with Mn(II) but, as for all other Class-I R2 proteins, displayed both radical generation and RNR activity when reconstituted with Fe(II).[116] The protein was also characterized structurally, showing that the protein shared structure and metal site architecture with other R2 proteins and that the di-Fe(II) loaded protein did react with molecular oxygen to produce an oxo-bridged diferric site. The Mn(II) substituted protein displayed a di-Mn (*cf.* mono-Mn) site and appeared not to be reactive with molecular oxygen, as observed also for other di-Mn proteins.[7–9,85,117–119] Because of these observations and the vast amount of data accumulated for iron-containing R2 proteins from both Class-Ia and Class-Ib, the existence of a manganese-dependent RNR was considered little more than an odd exception and very controversial.

This story recently took a drastic turn. In August of 2009 two papers appeared on the web just one day apart. The first reported the crystallization and preliminary X-ray analysis of the R2 from *C. ammoniagenes*.[120] The structure of this protein had been determined previously[85] but this crystallization report regarded the homologously overexpressed (*i.e.* in the native host) protein. X-ray fluorescence spectroscopy showed the presence of manganese but not iron, and anomalous difference maps showed the presence of a dinuclear manganese site. As such this report confirmed the earlier structural assignment as a di-Mn site in the manganese containing protein[85] and

suggested that the homologously overexpressed protein preferably incorporated manganese. Still, there was no firm link between the presence of manganese and radical or activity.

The second paper[121] was more revealing. This paper describes the EPR characterization of the R2 subunit from *Corynebacterium glutamicum*, a protein showing ~80% sequence identity to the *C. ammoniagenes* R2.[122] The protein prepared from the native organism contained 0.8 mol Mn per protein monomer but only 0.06 mol Fe, displayed a radical EPR signal and was enzymatically active. The saturation behavior and lineshape of the EPR signal also suggested the proximity of a paramagnetic metal site. Because it is most likely not possible to exchange metals while keeping the radical in the protein, this study indicated that the observed radical was generated by, or near a manganese site in the protein. Because previous studies had shown that purified Mn-loaded R2 proteins, including the highly similar *C. ammoniagenes* R2, does not react directly with molecular oxygen, together, these results suggested that there had to be a missing piece of the puzzle.

A few months later, this piece fell into place. In January of 2010 Cotruvo and Stubbe reported the biochemical and spectroscopic characterization of the *E. coli* Class Ib RNR[123] (*E. coli* encodes both a Class-Ia and a Class-Ib RNR system). In this study, using purified components, they show that the R2F (Class-Ib) protein associates with NrdI, an unusual flavodoxin present in the same operon. The Mn(II)-loaded R2F produced the tyrosyl radical and an active protein when incubated with molecular oxygen in the presence, but not in the absence, of reduced NrdI. When apo R2F was loaded with Fe(II), a radical and activity (albeit lower) could be generated by direct reaction with molecular oxygen. The presence of NrdI in this case lowered both radical content and activity. The authors proposed that reduced NrdI reacts with O_2 to produce HO_2^- that is then funnelled to the manganese site to act as the actual metal oxidant and that this reaction is repeated one more time to generate the high-valent metal site needed for tyrosyl radical generation. Attempts to produce the radical and activity by chemical oxidation of the di-Mn cofactor with H_2O_2 in the absence of NrdI proved unsuccessful, as also observed for the *C. ammoniagenes* Mn-loaded R2F.[116]

During the fall of 2010, two beautifully complementary studies appeared within a week of each other that provided a wealth of new information and firmly established the di-Mn R2 proteins (commented in ref. 124). Cox and coworkers[125] described the high-resolution structure and in-depth spectroscopic characterization of the holmologously overexpressed di-Mn containing *C. ammoniagenes* protein. The study shows that the active form contains an oxo- or hydroxo-bridged ferromagnetically exchange-coupled Mn(III)–Mn(III) dimer that is weakly coupled to the tyrosyl radical (Fig. 4). A mechanism for cofactor assembly *via* oxidation by peroxo-species and directed by conformational changes of the metal–ligand sphere was also proposed. In this study the authors also report the rapid and complete recovery of the radical in the hydroxyurea quenched protein by incubation with 10 μM H_2O_2 in the presence of the mediator methylviologen. This study was also further extended later.[126]

Just a week after the Cox study, Boal and coworkers[127] reported the crystal structures of Mn and Fe containing *E. coli* R2F as well as a number of forms of the complex between R2F and NrdI. The study describes significant conformational changes of the metal ligands depending on whether Fe or Mn was bound in the protein. The most fascinating results presented in this study are the complex structures showing a stretch of water molecules mapping out a path through a closed channel leading from the FMN cofactor in NrdI to the metal site. This channel may serve as the path for transferring the oxidant, generated by NrdI, to the metal site. This hypothesis is supported by the chemical properties of the channel and the fact that a ligand, possibly a peroxide species, is trapped in the channel in one of the crystal structures (Fig. 5). This study thus provides the structural basis for activation of the di-Mn cofactor in R2F.

The fact that all organisms encoding Class-Ib R2 proteins also appear to posses the NrdI gene (RNRdb, http://rnrdb.molbio.su.se/)[128] suggests the possibility that all Class-Ib R2 proteins may be able to use manganese together with NrdI for radical generation. Based on current knowledge, it also appears that these proteins are able to use iron *via* direct reaction with molecular oxygen to generate the radical. The question of which is the native cofactor, *i.e.* most important *in vivo*, is not firmly established and may of course also differ between organisms and environmental conditions. What is clear, however, is that manganese is certainly competent as a cofactor in the above Class Ib proteins. It is reasonable to assume that the di-Mn cofactor plays an important role *in vivo*. This appears almost certain for corynebacteria, which require Mn for growth and from which isolated R2F proteins almost exclusively contain manganese.[125] In addition there is data to suggest that the Mn–RNRs are important for growth during iron starvation or conditions of oxidative stress.[129–133] A key remaining question for this group is what is the actual metal site-coupled oxidant that generates the tyrosyl radical in the dimanganese proteins. One possibility is a Mn(III)–Mn(IV) metal site, in analogy to the radical generating Fe(III)–Fe(IV) intermediate X in diiron R2 proteins.[121,123] Other alternatives could possibly be a Mn(IV)–Mn(IV) metal site or some type of peroxo-coupled high-valent Mn metal site. The scene is set for a very exciting continuation of this story.

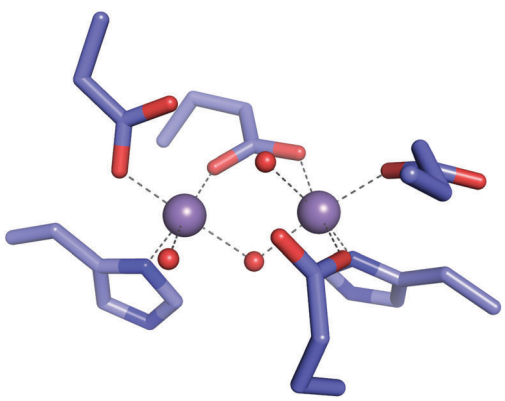

Fig. 4 Structure of the oxidized Mn(III)–Mn(III) metal site in *C. ammoniagenes* R2F. The metal coordination is very similar to what is observed in the structures of R2 proteins with Fe(III)–Fe(III) sites. The non protein ligands are assigned as a bridging oxo/hydroxo ligand and two terminal hydroxo/water ligands.

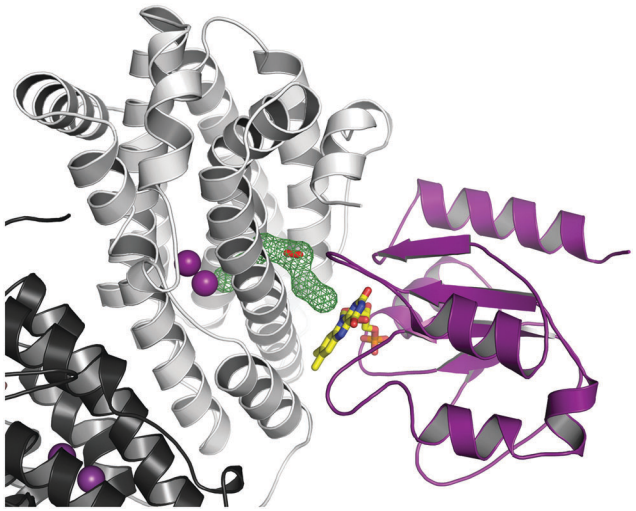

Fig. 5 Structure of the E. coli R2F-NrdI complex. For clarity, the figure is focused on one of the two R2F-NrdI subcomplexes (white and purple) in the actual $\alpha_2\beta_2$ complex (arranged as NrdI-R2F-R2F-NrdI), part of the second R2F monomer can be seen in black. The manganese ions are indicated in purple and the FMN cofactor in yellow. The closed path, putatively used for transfer of the oxidant from the FMN to the metal site is indicated with a green mesh. An exogenous ligand, assigned as a peroxo species, in the channel is indicated in red.

Emergence of new groups of R2-like proteins

In the paper describing the structure of the *C. trachomatis* R2c protein, a handful of sequences from other organisms were also identified to possess the same sequence features, *i.e.* showing significant overall similarity to R2 proteins but lacking the tyrosyl radical site and displaying four metal coordinating glutamate residues.[58] As sequence databases grew, more and more sequences could be added to this group. Recently, analysis of these sequences suggested that the group is actually further subdivided into two groups (Fig. 6), of which one appears to lack certain additional sequence features that were otherwise conserved in R2 proteins of all subgroups, such as a tyrosine residue located in the C-terminal tail of the protein, shown to be involved in the radical transfer between the R2 and R1 subunits.[134] The structure of a *Mycobacterium tuberculosis* protein belonging to this second group showed that this protein had an overall structural similarity to R2 proteins but displayed a drastic remodeling of the R2 protein scaffold to encompass a large ligand-binding cavity and a bound ligand, coordinating directly to the metal site (Fig. 7A).[134] Metal analysis of the protein expressed under different metal conditions showed that it always contained close to stoichiometric amounts of Mn and Fe and X-ray anomalous dispersion measurements of the protein crystals provided the first structure of the heterodinuclear cofactor and showed that the metal binding is specific with Mn occupying position 1 and Fe position 2 (Fig. 7B). The metal binding specificity thus appears to be strict in this protein, unlike in the *C. trachomatis* R2.[58,93,99]

There was also a very peculiar feature in that an unprecedented Tyrosine–Valine crosslink was present in the protein close to the metal site (Fig. 7A). Because the crosslink is deeply buried in the protein and the protein was heterologously overexpressed, the only plausible explanation for the formation of the crosslink is *via* a 2-electron oxidation by the metal site. Based on the structural features and the observed chemical potential of the metal site, this group of proteins was denoted R2lox (R2-like ligand binding oxidase) although the *in vivo* function is still unknown. Recently, a detailed bioinformatic study was performed, identifying the R2c and R2lox sequences present in the databases and comparing their pattern of sequence conservation with each other and the standard R2 proteins.[135] Interestingly, R2c and R2lox proteins appear predominantly present in pathogens, extremophiles, archaea and organisms isolated from chemically contaminated soil.

A protein that is structurally similar to the *M. tuberculosis* R2lox quietly appeared in the PDB database, PDB id 2oc5, deposited by The Joint Center for Structural Genomics (JCSG) (http://www.jcsg.org/). This structure shows a dinuclear metal site and an unknown ligand bound in a very similar fashion to the one in the *M. tuberculosis* R2lox (Fig. 8). The metals are assigned as two iron ions but it is not disclosed on what basis. Recently, it was shown that this protein catalyzes the last step of a microbial alkane synthesis pathway, converting a fatty aldehyde to its alkane or alkene analog.[136] Even though the exact chemical nature of the reaction or the metal cofactor of the protein is not conclusively defined, it is a striking example of how R2-like proteins appear in more and more functional contexts.

Conclusions and perspectives

The R2 proteins have been extensively studied for over four decades and were generally assumed to utilize only iron cofactors. Discoveries during the last few years have now firmly established that R2 proteins also employ heterodinuclear Mn–Fe and di-Mn cofactors. Unlike some proteins that can function with different metals[137,138] the differences in redox properties between Mn and Fe require adaptations of the R2 protein component or the radical generation mechanism if one metal is to functionally substitute for the other. In the Class-Ib R2 proteins both di-iron and di-manganese cofactors can produce catalytically competent enzymes. However, radical generation in the dimanganese-loaded protein requires an additional protein, NrdI, to produce a different

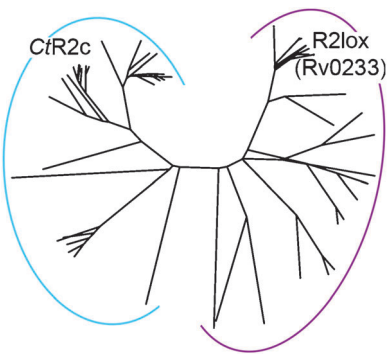

Fig. 6 Phylogenetic tree of a number of R2-protein homologues with sequences that lack the otherwise radical harboring tyrosine. The locations of *C. trachomatis* R2c and *M. tuberculosis* R2lox (Rv0233) are indicated.

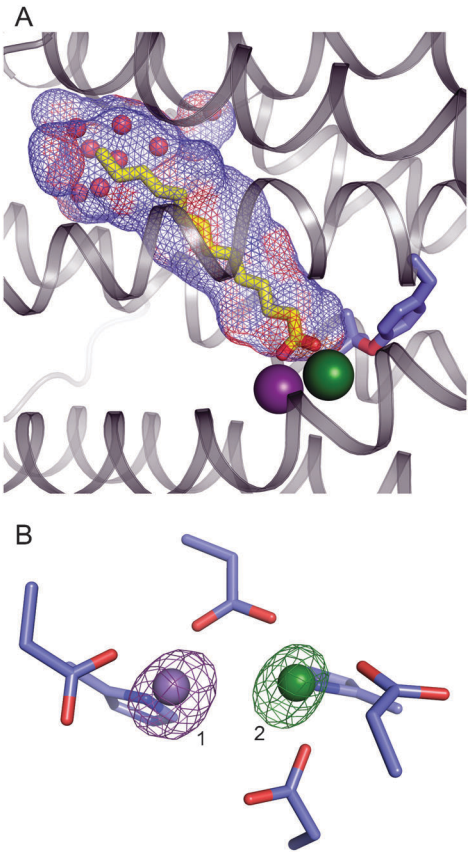

Fig. 7 (A) Ligand-binding cavity and bound ligand in the R2 lox protein. Manganese ion in magenta, iron in green. Tyr–Val covalent crosslink at the metal site shown in blue sticks. (B) Anomalous difference electron density for manganese (Magenta, left) and iron (Green, right).

primary oxidant for the reaction. NrdI appears to be present in all organisms encoding Class Ib R2 proteins; this, together with the fact that the R2F proteins from Corynebacteria appear to contain in essence only manganese when isolated from their native host, strongly suggest that the dimanganese form of the protein is important *in vivo*.

The biochemical rationales behind the use of manganese in R2 proteins are also beginning to be revealed. Interestingly, the background appears different, but possibly with a similar foundation. In the R2F, di-Mn, case it appears as a backup system for the crucial RNR reaction under particular conditions, such as limiting Fe.[129–132] The fact that these proteins, at least in some cases are upregulated under various kinds of oxidative stress also points to a possible adaptation for a more resistant cofactor against, *e.g.* reactive oxygen species.[133] For Class Ic proteins with a heterodinuclear Mn–Fe cofactor, it seems like the reason is to utilize the different redox properties of iron and manganese to tune the resulting redox potential of the oxidized di-metal cofactor to allow reversible radical transfer directly from the metal site in R2, thus bypassing the need for a tyrosyl radical. The tyrosyl radical is believed to be a target for direct reactions with radical scavengers, such as NO, and the underlying reason for avoiding the tyrosyl radical may be to produce a system that is more resistant to radical scavengers.[58,107] In both cases, it appears that Nature has devised a truly bioinorganic workaround to ensure that production of deoxyribonycleotides is maintained under growth conditions that would severely impair standard R2 proteins, such as limiting iron availability or the presence of potential radical scavengers. The fact that the Mn–Fe R2c and R2lox proteins are primarily found in extremophiles and pathogens[135] agrees with this hypothesis. These organisms are expected to be particularly exposed to potential radical scavengers such as reactive nitrogen and oxygen species, produced by the immune response of the host or an especially aggressive growth environment. Unlike in di-iron R2s, the Mn–Fe R2 reaction involves a Mn(IV)–Fe(IV) intermediate. This observation, together with the ligand-binding properties of the recently discovered R2lox group of proteins, suggests that the heterodinuclear cofactor can also be used for two-electron oxidations. The exact nature of the metal cofactor of the R2-like protein of the recently described microbial alkane synthesis pathway[136] remains to be determined. Still, this protein, in addition to the Mn–Fe R2lox group, establishes R2-like proteins as di-metal substrate-converting catalysts with interesting implications for both academic and industrial fields.

From a broader perspective, the developments regarding the metal dependence in the R2 family is an educational example of how reductionist scientific reasoning can be misleading. Bioinorganic chemistry, where protein catalysts interface with inorganic ones, is a field where particular care should be employed. As biochemists, we tend to see a metalloprotein as a pre-defined entity, the protein and its native metal cofactor, that will perform a defined function. For organometallic chemists, the concept of metal exchange in catalysts is certainly not new and the statement that a particular chelating molecule will obtain different catalytic properties depending on what metal one chooses to incorporate in it is undisputed and almost trivial. From a chemical point of view, it is not difficult to accept that protein chelators will also show this behavior and it is known that catalytic activity, of the same or a different reaction, can be obtained with different metals also in protein catalysts. Thus, bioinorganic chemistry introduces an additional layer of complexity when studying protein function.

An important feature of metalloproteins is that the properties of the catalyst is decided, to a very large degree, by a moiety that is easily exchangeable compared to changing protein properties by introducing mutations in the genes. In addition, because metal specificity is never absolute, subpopulations of proteins with different metals or without an incorporated metal will always exist. From an evolutionary perspective, Nature of course benefits from this fact by continually sampling the potential metabolic and catalytic contributions from protein subpopulations with (mis)incorporated metal sites. Promiscuous functionality in protein catalysis is believed to be a key component of the evolution of new functions.[139] Non-strict metal incorporation can be used as a facile way to create catalytic diversity that can later be captured and reinforced by gene duplication and mutational changes in the protein to produce a catalyst with a new main function or performing the same function using a different mechanism or cofactor. The promiscuity in terms of mechanism and metal cofactor usage in R2 proteins as well as the R2-like proteins of

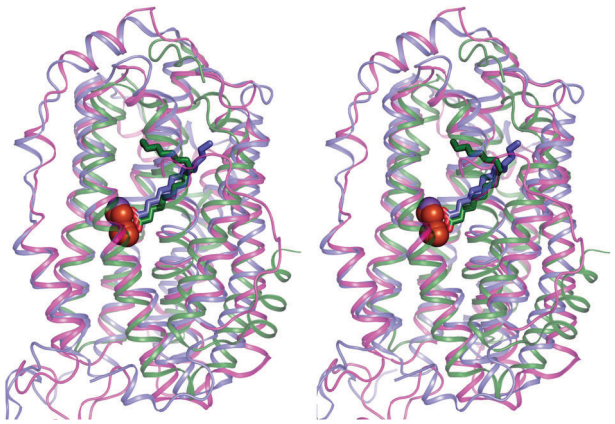

Fig. 8 Stereo figure showing structural similarities between the *C. trachomatis* R2c (magenta), *M. tuberculosis* R2lox (blue) and *Prochlorococcus marinus* alkane synthesizing protein (PDB id: 2oc5) (green). Note the extensive overall structural similarity and the very similar ligand coordinating properties of R2lox (blue) and the *P. marinus* protein (green).

different function that have been discovered during the last few years are likely direct examples of these chemical and evolutionary principles in action. Even though many of the specific adaptations are probably uncommon and possibly even unique to the R2-like family, it is reasonable to assume that there are still a great number of promiscuous functions left to discover among even well-studied metalloprotein catalysts. These functions may be of different levels of significance *in vivo*, but still important for the systems biochemistry of the organism and our understanding and utilization of metalloprotein catalysts.

The inherent properties of metalloprotein catalysts described above highlight the importance of metallomics to obtain a global view of metalloproteomes. When assigning the metal content of individual proteins one should also consider the relative amounts of different metals and not just the dominating one. To be authoritative, this information would have to be obtained from the protein produced in its natural context and at native expression levels. Such a database would be of utmost value to the field. In theory, this information could be obtained by obtaining whole-cell samples of various organisms, growth conditions, tissues in the body *etc*., followed by isolation of all protein species and quantification their metal content. Experimentally this is a formidable task, and even though high-throughput methods for metal analysis in proteins are starting to be developed, there is still a great need for novel methods and approaches to analyze proteins with low natural levels of expression.[140–146]

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