

Ribonucleotide reductases: metal and free radical interplay

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Abstract

In the cell, the only de novo biosynthetic pathway to desoxyribonucleotides is by reduction of their ribonucleotide counterparts. This reaction is catalysed by the ribonucleotide reductases. Three classes of these metalloenzymes are known which all resort to the same synthetic strategy: radical activation of the substrate by means of a protein free radical followed by a

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three-electron reduction step. The nature of the essential protein free radical and its generation differ widely from one class to another. In this paper, the characteristics of the different metal and radical interplay of each class are reviewed. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Ribonucleotide reductases; Radical activation; DNA

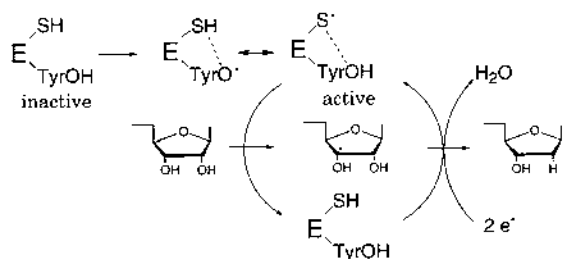
1. Introduction

All living organisms depend on the elaboration and transfer of genetic information contained in DNA. The four deoxyribonucleotides which are the building blocks for DNA synthesis and repair are synthesized *in vivo* by reduction of their ribonucleotide counterparts. The enzymes which catalyse this reaction are the ribonucleotide reductases.

Ribonucleotide reductases have evolved under three different classes according to the external growth conditions of the organism considered but they all employ the same chemistry to achieve the difficult stereospecific reduction of the 2'-hydroxyl of a ribonucleotide. The chemical answer was the use of a protein free radical to activate, by one-electron oxidation, the ribonucleotide substrate. A subsequent three-electron reduction step then yields the deoxyribonucleotide and recycle the starting free radical. (Scheme 1).

Radicals are highly reactive species and ribonucleotide reductases had to find a way to control and modulate their reactivities. They solved this problem by employing a metal center to generate and stabilize inside an hydrophobic buried site a free radical on a specific residue of the protein polypeptide. A second trick was to move away the radical from the actual site of reduction and to provide a switch between the two. Finally to solve the specificity problem of four different substrates for only one active site they selected a beautifully sophisticated mechanism based on allosterically-controlled conformational changes.

This paper deals with the three classes of ribonucleotide reductases and will specifically address what makes them different from one another, that is, the nature, the structure and the reactivity of their metallic centers and the generation and the



Scheme 1. The class I ribonucleotide reductase reaction.

characteristics of their stable free radical. Once the radical in the enzyme is generated, the chemistry which takes place is strikingly similar irrespective of the class considered and several excellent papers [1–3] are available on the radical transfer reactions to the active site and on the chemistry which takes place there.

2. Class I

Class I ribonucleotide reductases are by far the best studied of these enzymes. They are found in all eukaryotic organisms (animals and superior plants), in some procaryotes and viruses and a few bacteriophages. The prototype for this class is the enzyme from *E. coli* encoded by the *nrdAB* genes on which structural and mechanistic studies have accumulated in the last 30 years. A subclass of this group (class Ib) has recently been described (see below).

2.1. Class I: the large subunit

The *E. coli* enzyme is an heterotetramer $\alpha_2\beta_2$ made of two homodimers called R1 (α_2) and R2 (β_2) [4]. The large subunit, α_2 , is encoded by the *nrdA* gene. The structure of the α_2 subunit has been solved at 2.5 Å resolution [5]. It is the only α_2 structure solved to date. The 761-residues polypeptide chain is arranged into three domains: one mainly helical N domain, one α/β barrel domain comprising ten antiparallel β strands (480 residues) and a 70-residue domain arranged in an $\alpha\beta\alpha\beta$ structure at the C terminus. The active site is located at the centre of a deep cleft across the subunit between the N terminal and barrel domains and exhibits the 3 cysteines which, on the basis of biochemical [6,7] and mutant studies [8–10], had been previously proposed to be directly involved with the reduction of the substrates. The α/β barrel is wide enough to accommodate the insertion of a loop like a finger in its centre. One of the conserved cysteines, 439, sits on the tip of this finger. The two other conserved cysteines are separated by hundreds of residues but held close by their location on two adjacent β strands. Actually, in the original structure, these cysteines occurred as a disulfide located on the bottom face of the ribonucleotide substrate [5]. Soaking the crystal into DTT allowed the determination of the reduced form of the protein [11]. In the reduced form (Fig. 1), Cys225 has moved 2 Å away from its position in the oxidized state and Cys462 is now deep inside the interior of the protein at about 6 Å creating an opening which facilitates the binding of the substrate and the positioning of the C3' at van der Waals contact (3.4 Å) of the Cys439. The latter, proposed to be the radical initiator of the reaction, is lying on the top face of the ribose. Also noticeable are the hydrogen bonds to the O3' with two conserved residues: Glu441, providing the only charge of the site and Asn437, occupying a key position in the centre of the active site. This is the only redox-active thiol protein known to exhibit such a dramatic reorganization upon redox changes.

Four other conserved residues play a key role. The two cysteines Cys754 and Cys759 are not visible in the structure probably because of their location in flexible

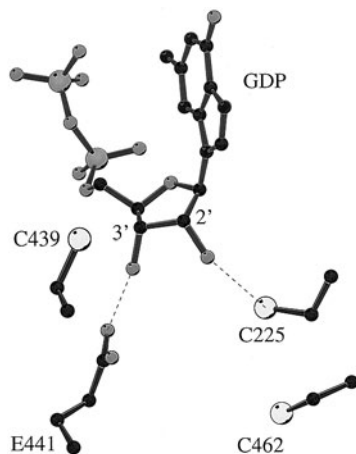


Fig. 1. The nucleotide active site of the *E. coli* protein R1 (from Ref. [11]).

parts of the polypeptide chain. They are in the typical topological relationship of dithiol proteins and shuttles the electrons from the external electron donor (thioredoxin or glutaredoxin systems [12]) to the redox active site thiols. The two tyrosines Tyr730 and Tyr731 H-bonded together and to the Cys439 are postulated to be part of a radical transfer chain between the stable radical Tyr122 on R2 and this Cys439 on R1 [13].

2.2. Class I: the small subunit

The small subunit R2 is encoded by the *nrdB* gene as an homodimer of 375 amino acids. It carries a μ oxo, μ carboxylato bridged diferric center and in its vicinity (5 Å) a tyrosyl radical when active. Because of these two centers, the protein exists under several redox forms [14]. One-electron reduction of the radical leads to the *met* form. A further one-electron reduction leads to a quite unstable *semi-met* form which has a mixed valent Fe(II)Fe(III) binuclear center and a tyrosine and is thus EPR-active ($g < 2.00$). The completely reduced form is also EPR-active ($g = 14-16$) and carries a dimer of Fe(II). In addition, a form devoided of any metals (apo form) has been characterized. Among these, three forms have been crystallographically solved: the *met*, the reduced and the apo forms.

The structure of *met* R2 has been solved for two proteins, that of *E. coli* [15,16] (to 2.2 Å resolution) and that of the mouse enzyme [17] (to 2.3 Å). The two structures can be superimposed with an r.m.s. deviation of 1.6 Å for 262 C α atoms. The subunit is mainly helical in both structures. In the case of the *E. coli* enzyme these helices are unusually long and set it apart from other dinuclear iron proteins of this class [18] such as methane monooxygenase (MMO).

In the *met E. coli* structure (Fig. 2), the two iron atoms are separated by 3.3–3.4 Å and linked by one oxo bridge and one carboxylato bridge (Glu115). The dihedral angle of Fe–O–Fe is about 130°. Each iron is hexacoordinated having one histidine

ligand (His118/241), one water molecule, sharing the oxo and carboxylato bridges and differing for the last two coordination sites. The Fe(1), close to the tyrosine Tyr122 has a bidentate carboxylate (Asp84) whereas Fe(2) has two monodentate carboxylates (Glu204 and Glu238). All ligands of iron are part of a 4 helix bundle in which the di-iron axis is oriented parallel to the long axis of the bundle. The arrangement of the ligands according to the helices (D/E, ExxH, E, ExxH related to the helices B, C, E, F respectively) is conserved in a class of iron oxo proteins comprising MMO, stearyl-acyl carrier protein $\Delta 9$ desaturase, toluene hydroxylase, phenol hydroxylase and alkene hydroxylase [18].

In the second coordination sphere, other conserved residues are found. The tyrosine 122, which in the active form is the essential radical, is located 5.3 Å away from the Fe(1) and has the same conformation as the one deduced in solution of the active form from EPR hyperfine couplings of the radical with the β protons of the tyrosine nucleus [19]. This site is surrounded by an hydrophobic pocket consisting of non polar conserved residues Phe208, Phe211 and Ile234 all of which are proved by site directed mutagenesis to be essential determinants for the stability of the radical [20,21]. Away from the tyrosine site, several residues are conserved including a His, Asp, Trp triad connecting Fe(1) to the surface of the protein by an extensive network of hydrogen bonds.

In the mouse protein, solved at 2.3 Å resolution at pH: 4.7, only the Fe(2) site is partially occupied [17]. The iron is pentacoordinated in a bipyramidal coordination with three Glu and one apical His, the other apical site is vacant. The structure also shows the existence of a narrow channel near the Fe(1) site proposed to be the binding site of O₂. Small changes in the structures likely explain the differences in stability of the metal in the two species [22,23].

The crystal structure of the reduced R2 of the *E. coli* enzyme has been determined to 1.7 Å resolution [24]. The two tetracoordinated iron atoms are separated by 3.9 Å and linked by two carboxylato bridges (Glu238 and Glu115).

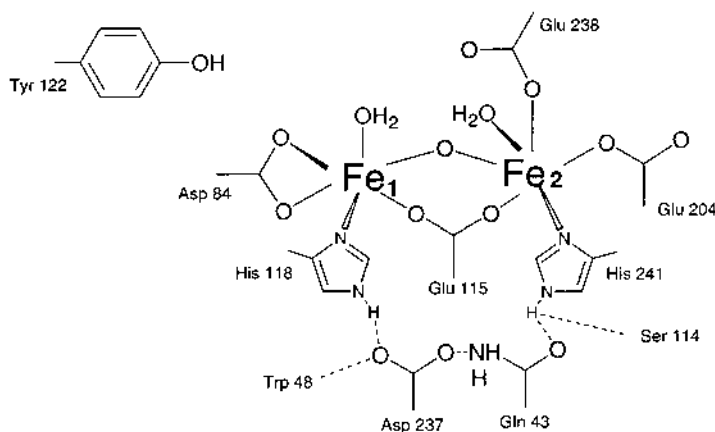


Fig. 2. Structural representation of the diferric-oxo site of *E. coli* protein R2 (from Ref. [15]).

The changes in coordination resulting from a shift in the carboxylate binding mode likely tunes the redox potential of the center and thus controls the binding of oxygen [25]. This carboxylate shift is probably triggered by the movement of Fe(2) away from Fe(1) which in turn forms an unusually short (1.83 Å) bond to Asp84.

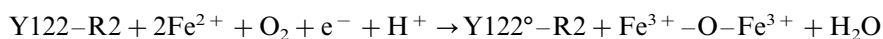
The structure of the reduced center reveals that its net charge is zero. This feature is also found for the other crystallised forms of R2 (apo, *met*R2 and Mn–R2 [26]) and suggests that any electron transfer to or from the center must be accompanied by a transfer of a proton. The structure of the apo form is virtually superimposable to that of the reduced form with four protons substituting for the two iron atoms [27].

Attempts have been made to obtain the structure of the *semimet* form of the R2 protein from *E. coli* by applying intense synchrotron X-radiation at 100 K to a crystal in the *met* form [24]. However these conditions led to the reduced form of the protein only. The mixed valence state of the *E. coli* enzyme is unstable and was generated in a very low yield under γ -ray radiation [28] or by chemical reduction with strong reductants [29]. On the contrary, mixed-valent R2 of other species could be obtained by γ -ray irradiation of frozen solution or mild reducing chemical systems with yields of 10–30% as quantitated by the $g < 2.0$ EPR signals [30]. These differences in reactivity are readily rationalized by cyclic voltammetry studies showing, for the *E. coli* enzyme, a two e-process with a lower midpoint potential (–115 mV) compared to two well defined waves at more positive potentials for the mouse R2 or for the MMO hydroxylase protein [31,32].

2.3. Class I: the formation of the tyrosyl radical

Class I reductase activity rests on the presence of the TyrO[•] radical on the small subunit. In recent years, an impressive collection of data on the generation of this radical has appeared in the literature. Once again, the *E. coli* enzyme was the preferred target of these studies along with several point-mutated analogs.

The stoichiometry of the activation reaction has recently been reestablished [33]:



The two ferrous ions and the oxidisable Tyrosine each provides one electron. The fourth electron needed to achieve O₂ reduction comes from either excess Fe²⁺ or ascorbate in vitro.

This reaction has been investigated by stopped-flow UV-vis, rapid freeze quench (RFQ)-EPR and RFQ-Mössbauer spectroscopies [33–35]. Two intermediates have been identified depending on the reductant/R2 ratio. Under conditions of limiting reductant, an early intermediate is detected at 565 nm, the nature of which is still controversial. It was first proposed to be a μ -1,2 peroxo diferric species [36] but later on, was reevaluated as a tryptophan cation radical [35]. However, the lack of temporal correlation between the different rapid techniques leaves its identity open. A second intermediate, called compound X, which is the only one detected in presence of excess reductant, has been fully characterized as a spin-coupled Fe(III)/Fe(IV) center on the basis of its Mössbauer parameters ($\delta\text{Fe(1)} = 0.56(3)$

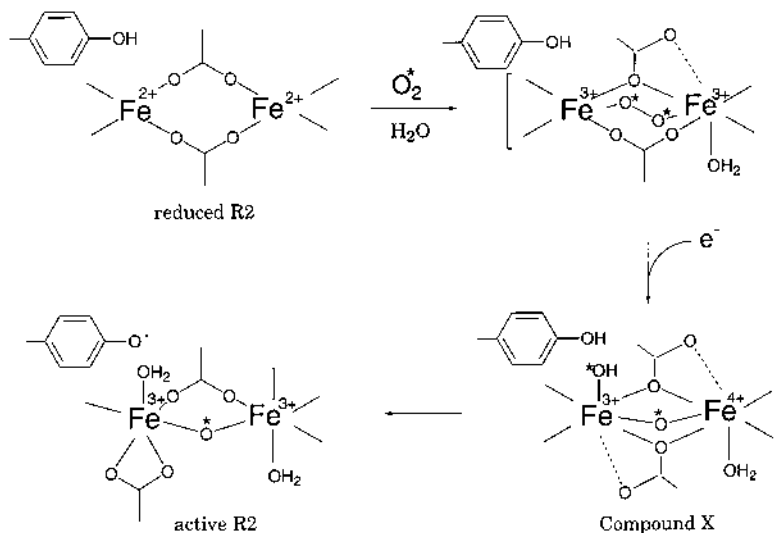


Fig. 3. Mechanistic proposal for the formation of the Tyrosyl radical.

mm s^{-1} , $\delta\text{Fe}(2) = 0.26(4) \text{ mm s}^{-1}$) and of the Q-band ENDOR of the ^{57}Fe -reconstituted protein showing a strong hyperfine anisotropy for Fe(2) inconsistent with the diferric + radical structure initially proposed [37,38].

In an effort to increase the lifetime of this intermediate, the mutant Y122F has also been studied and shown to exhibit the compound X-characteristic $S = 1/2$ EPR signal which broadened with the use of $^{17}\text{O}_2$, H_2^{17}O and ^{57}Fe [37]. ^1H and ^{17}O ENDOR spectroscopy have led to the final picture of X as being a $\text{Fe}^{3+} \text{Fe}^{4+}$ dimer with one oxo bridge and a water molecule [39]. Using a protocol with ^{56}Fe and ^{57}Fe in reconstitution experiments, it has been possible by RFQ-Mössbauer to show a higher affinity of the Fe(2) binding site (the site away from the tyrosine) for iron. Also, these experiments have shown that the Fe^{4+} character of X mainly resides on this Fe(2). The authors speculated that a more reducible Fe(1) site may have evolved so as to direct the outcome of the reaction to the one electron oxidation of the tyrosine nearby [40].

Recently the structure of X has been assessed by X-ray absorption spectroscopy and shown to contain 2 irons in close proximity (2.49 Å) and a strong Fe–O interaction at 1.8 Å consistent with the presence of a μ -oxo bridge [41]. The data are consistent with a triple bridge comprising two monodentate bridging carboxylates and one μ -oxo bridge. Resonance Raman spectroscopy served to demonstrate that, in the diferric end product, the μ -oxo bridge is derived from O_2 gas [42] (Fig. 3).

As mentioned above, the iron center of R2 bears considerable analogies with several other non heme iron proteins and among them, most notably, the methane monooxygenase hydroxylase component (MMOH). In that case, the di-ferric center is converted to compound Q, containing an $\text{Fe}^{4+} \text{Fe}^{4+}$ center, which is the actual

two-electron oxidant of the unactivated hydrocarbon substrate. The close analogies between the two systems have prompted the search for the structural determinants able to direct the outcome of the O₂ activation toward one or two-electron chemistry.

Thus, the mutant Phe208Tyr, which brings an oxidizable tyrosine into the hydrophobic pocket surrounding the Tyr122 site, was found to partition between the normal one-electron Tyr122 oxidation and *ortho*-hydroxylation of Tyr208 leading to an iron chelate of DOPA as the major product. [43]. The latter reaction is completely suppressed in the presence of large amounts of ascorbate which increases the rate of electron injection in the system [44]. Moreover, the double mutant Phe208Tyr/Trp48Phe, for which the H-bonded connection path from the protein surface to the cluster inside is broken, leads to an increased formation of the DOPA derivative and, in this case, ascorbate has no effect on the outcome of the reaction [44]. These data suggest, in R2, the existence of a very reactive intermediate, formally analogous to the **Q** intermediate of MMOH, which is reduced by one electron at such a rate that it eludes detection by fast kinetic techniques and prevents a two-electron oxidation from occurring.

The close correspondence between the two systems is also evident from parallel studies on the early intermediates. On mixing reduced MMO with O₂, a transient visible absorption appears at around 700 nm and has been assigned to an intermediate called ‘H-peroxo’. Its Mössbauer parameters are consistent with a diamagnetic μ -(η^1 : η^1) peroxo diiron(III) ($\delta = 0.66 \text{ mm s}^{-1}$, $\Delta E_Q = 1.58 \text{ mm s}^{-1}$) formulation [45]. No such intermediate could be detected in the case of R2. However, by single point mutation (R2-Asp84Glu) substituting the aspartate of wild type R2 by the corresponding glutamate found in MMO, a new intermediate is now detectable on the route of TyrO° formation. Its Mössbauer and resonance Raman parameters are strikingly similar to MMOH-‘H-peroxo’ [46,47]. Conversely, low-temperature radiolytic reduction converts the **Q** intermediate of MMO to a mixed valent state analogous to R2-X [48].

The activation reaction results in a deprotonated tyrosyl radical. This has been shown by resonance Raman ($\nu_{(C-O)} = 1498 \text{ cm}^{-1}$ invariant in D₂O [49]), by ENDOR spectroscopy showing the absence of any coupled proton [50], by the high g_x value (2.0078) observed in the EPR at 245 GHz [51] and by the sharp visible absorption at 410 nm.

The microwave saturation behavior of the X-band EPR signal indicates that the two paramagnetic centers are weakly interacting [52]. EPR saturation recovery studies at variable temperature have been used to quantitatively assess the exchange parameters and dipolar coupling energy [53] and have shown significant differences among different centers. The hyperfine couplings observed at low microwave frequency are related to the coupling between the unpaired spin with the nuclear spin of the adjacent β protons. In the *E. coli* enzyme they indicate a dihedral angle of about 30° between the plane of the aromatic ring and the out of plane β proton. On the other hand, the tyrosyl radical in mouse or *A. thaliana* R2s are more like the one found for Y_D of the PSII having a dihedral angle close to 80° [54]. The saturation and relaxation behavior of the signal point to a stronger magnetic interaction for the latter enzymes possibly explaining their lower stability.

The formation of the essential TyrO° radical thus encompasses binding of Fe to the apo form followed by O₂ interaction and a succession of fast steps leading to the formation of a stable tyrosyl radical and a μ-oxo diferric center. This set of events has conclusively been established with chemical reductants. The question then arises of the validity of this sequence when using the physiological reductants of the system.

It is well established that the inactive *met* R2 form can be activated in vitro by incubation with an enzymatic reducing system comprising a NADPH:flavin oxidoreductase, encoded by *fre*, free flavins, SOD and catalase with the obligatory presence of a poorly defined protein fractions called 'b' [55,56]. The latter can be substituted by Fe²⁺. In aerobic conditions, the 'b' fraction shuttles the electrons from NADPH to the metal center. Little is known about this reaction but studies with the mutant Tyr356Ala localized on the flexible part of the C terminus indicate that this residue may provide an 'entry' for the electrons to reduce the di-ferric center via the same H-bonded pathway proposed to be operative for the R2/R1 radical transfer [57]. Consistent with these findings is a recent report on the activation of the mouse R2 protein showing that the reduction of the binuclear iron is dependent on a H-bonded path involving Asp266 and Trp103 of the aforementioned triad [58].

2.4. The subclass Ib

Recently, a new group of enzymes was classified as the subclass Ib [59]. They were found in aerobic bacteria such as *L. lactis*, *E. coli*, *S. typhimurium* and *M. tuberculosis*. They are encoded by the genes *nrdEF* which, excepted for *M. tuberculosis*, are not normally expressed under usual growth conditions. Three main features distinguish these enzymes from the class Ia: first, the large subunit lacks the 50 first aminoacids of the sequence implying that they do not have the allosteric activity site of the class Ia; second, the small subunit, while presenting strong sequence homologies with Ia R2, has a perturbed site at the essential tyrosine resulting in a TyrO° EPR signal showing hyperfine structure almost identical to the Y_D^o of PSII [60]; third, the class Ib has its own specific redoxin system related to glutaredoxin as the external electron donor for the reduction step [61].

3. Class II

Class II ribonucleotide reductases are B₁₂-dependent enzymes. They are found in aerobic and anaerobic organisms among both bacteria and archae. Until recently the enzyme from *Lactobacillus leichmanii* was the only fully characterized representative of this class [62]. In the past two years 4 other members of this class have been characterized and found rather distantly related to the *Lactobacillus* enzyme [63–65]. The class II RNRs are monomeric or dimeric proteins of 80–90 kDa depending on coenzyme B₁₂ for the reduction of nucleoside di or triphosphates.

No three-dimensional structure is available. However, at variance with the *L. leichmanii* enzyme, the RNR from the hyperthermophile *P. furiosus* has significant sequence homologies with both class I and class III enzymes [63]. This was utilized to build a model of the protein (Fig. 4). Interestingly, the model exhibits a cleft large enough to accommodate a B₁₂ molecule in a close proximity to the cysteine residue predicted to be the radical initiator of the reaction. Only two crystal structures of B₁₂-dependent proteins are known, for the cobalamin domain of methionine synthase and methylmalonyl-coenzyme A mutase [66,67]. In each case a His–Asp–Ser triad has been shown to be present at the B₁₂ binding site. Actually this His displaces the benzimidazole ligand of the coenzyme and is postulated to

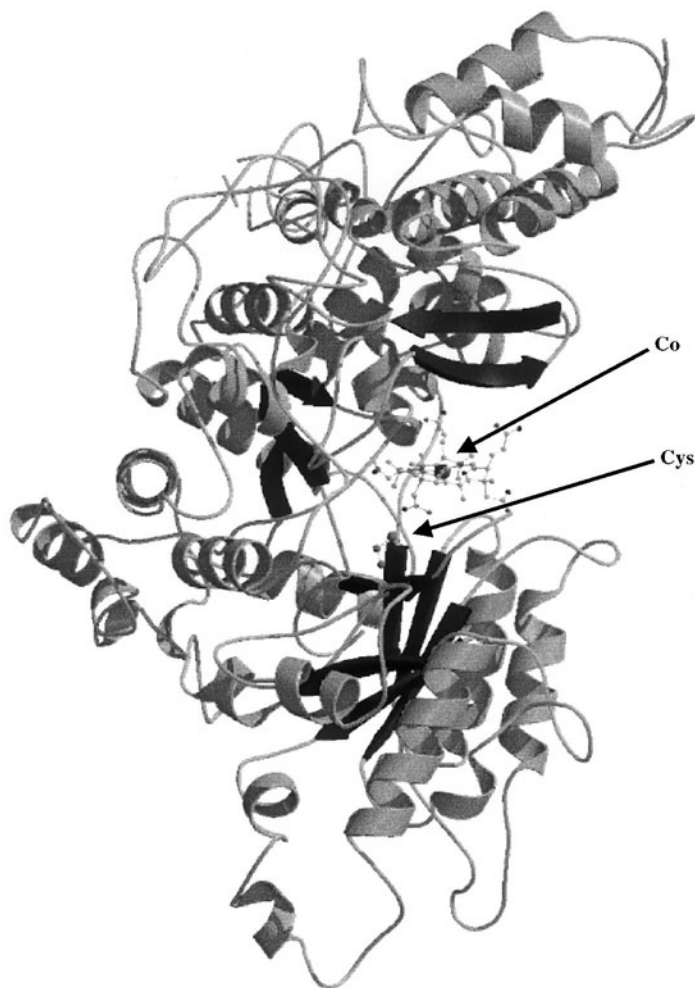
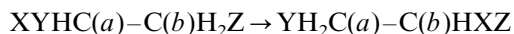


Fig. 4. Modelled tri-dimensional representation of the B₁₂-dependent class II ribonucleotide reductase from *Pyrococcus furiosus*.

ultimately control the reactivity of the cofactor toward homo or heterolytic Co–C bond cleavage. In the present *P. furiosus* model, no sequence of this kind could be found.

With maybe the notable exception of RNR, all B₁₂ dependent enzymes catalyse a 1,2 rearrangement such as:



This reaction is supposed to occur by an hydrogen abstraction at C(*b*) from the adenosyl radical formed by homolysis of the Co(III)–C bond followed by X migration on the adjacent carbon and formation of the product by back oxidation of the intermediate 5'-deoxyadenosine.

In the case of the RNR from *L. leichmanii*, the reaction has been studied in detail and, in contrast with the other B₁₂-dependent enzymes, a full release of ³H from labeled AdoCbl has been found [68,69]. This reaction has served as a diagnostic reaction for the class II enzymes. The release of the label to the medium implies the existence of a secondary radical site which has been recently demonstrated to be a cysteinyl radical by RFQ EPR [70]. To show this, the overexpressing recombinant cells were grown under conditions that allowed incorporation of β-(2H)cysteine and the EPR signals observed both for the exchange and for the reduction reaction was compared with the one obtained with the non labeled protein. In both cases a RFQ EPR *S* = 1/2 signal appears which, with the labeled enzyme, exhibited narrower hyperfines couplings with the Co nuclear spin. This implies the existence of a cysteinyl radical in a weak interaction with the cobalt atom. The lifetime of the intermediate is consistent (1) with the kinetics of appearance of Co(II) independently measured by stopped flow UV-vis absorption (2) with the quantitation of the 5'-deoxyadenosine formed by rapid acid quench and (3) with the quantitation of the unpaired spins by EPR.

The RNR from *L. leichmanii* has no sequence homologies to either class I or II except for a limited stretch on the N terminal part of the polypeptide. Nevertheless, as for the class I enzymes, it was possible to show the presence of five cysteines playing exactly the same role as in class I, namely every cysteine mutant behaved the same as for the class I enzyme thus embodying the firm basis of the mechanistic proposal [71].

4. Class III

Class III ribonucleotide reductases are found in facultative and strict anaerobic microorganisms. This class has been discovered in the late 1980's when it became clear that the activation of class I enzymes required oxygen. To the question of how anaerobic organisms obtained the dNTPs for DNA chain elongation and repair, the group of Reichard and Fontecave in Stockholm could show that these organisms use an enzyme different from those previously known at that time and employing specific organic and metallic cofactors [72]. This class has been most studied in anaerobic *E. coli* [73], which is considered as the prototype of this class, and also

in the bacteriophage T4 [74]. Up to now, there are more than 12 sequences known from facultative or strict anaerobes found in bacteria and archae (methanogens).

The *E. coli* enzyme is an $\alpha_2\beta_2$ tetramer which resembles the general organization of class I enzymes. However, there are important differences. It is encoded by the *nrdDG* genes which are part of an operon containing an unidentified ORF upstream of the *nrd* genes. The expression of these genes is regulated by the *fnr* system [75]. Under anaerobic conditions however, the two proteins are evidently not expressed at the same level suggesting a more complex regulation system.

4.1. Class III: the large subunit

The α_2 large subunit (2×80 kDa), encoded by the *nrdD* gene, does not contain any organic or metallic cofactor [76]. It shares at the N-terminus a region of limited homology with class I and II enzymes. This region is thought to contain the allosterically regulated overall activity site. Accordingly, the enzyme has been shown to employ regulation mechanisms similar to those of class Ia and II [77]. On the C-terminus, the polypeptide contains a cysteine-rich region with a sequence CX6CX2C not obviously related to any iron-sulfur center. In the neighbourhood of this sequence a pentapeptide RxxGY is conserved in all class III RNRs and other glycy radical enzymes such as the pyruvate formate lyase of *E. coli* [78] and the benzyl succinate synthase of *T. aromatica* [79]. This glycine (681 in the *E. coli* sequence) is a radical when the enzyme is active. This was unambiguously shown by three sets of experiments: *first*, a recombinant *E. coli* cell containing a multicopy plasmid coding for *nrdDG* genes was allowed to grow on a minimal medium containing glycine labeled on C2 either with ^2H or ^{13}C . EPR of the whole cells or of the reactivated purified enzymes shows the drastic and expected changes in the multiplicity of the signal. With non labeled glycine, the signal is a doublet ($a_{\text{H}} = 1.4$ mT) resulting from the coupling of the unpaired spin with the nuclear spin of a proton. In contrast, with the deuterated glycine, the signal appears as a singlet and the ^{13}C -labeled glycine as a complex signal with large hyperfine couplings due to the presence of the ^{13}C [80]; *second*, the mutants Gly681Ala, Cys680Ser and Tyr682Phe have been constructed and compared to the wild type both for their reductase activities and their radical contents. The two mutants Cys680Ser and Tyr682Phe showed reduced but still significant reductase activity (50 and 6.6%, respectively). Accordingly, these activities could be correlated to the EPR signal amplitude. On the other hand the G681A had no activity nor showed any significant EPR signal [80]; *third*, class III enzymes are extremely sensitive to oxygen. In fact, this extreme reactivity towards O_2 is apparent only when the enzyme is active. Indeed, the Gly $^\circ$ radical was shown to instantaneously react with oxygen leading to an irreversible reaction in which the backbone polypeptide is cleaved. This reaction was used to identify by ESMS the size of the 2 fragments produced. The results are in excellent agreement with a predicted cleavage between Cys680 and Gly681 [81].

At variance with class I and II enzymes, the localization of the putative cysteines of the active site is still unclear. Preliminary mutant studies done on the T4 enzyme indicate that, among the seven cysteines of the sequence, the cysteine adjacent to

the radical is not essential, the two cysteines located at the N-terminus (Cys79 and Cys290 in the T4 numbering) are not involved in Gly^o formation even though the mutant proteins are not active, the four remaining, close to the radical site, are each essential for Gly^o formation [82]. It remains to analyze these results more in depth to decide if the class III enzymes use the same set of cysteines as for the other classes.

4.2. Class III: the small subunit

The small subunit has been shown to be an homodimeric protein (2×17.5 kDa) only when complexed to α_2 [83]. The β protein is encoded by the *nrdG* gene. It contains an iron sulfur center [84,85] which is directly involved in the formation of the glycy radical on the α subunit. Its primary sequence [86] contains five cysteines (Cys19, Cys26, Cys30, Cys33, Cys96) of which the middle three are conserved in a limited number of proteins (biotin synthase [87], lipoate synthase [88], *fnr* regulatory protein [89]...). Interestingly, some of these proteins have been shown to contain unusual iron sulfur centers. Another striking peculiarity of unknown significance is the repeat of the GluCysProGly peptide for the two cysteines, Cys30 and Cys96.

Overexpression of the β peptide was achieved using the Studier overexpressing system in BL21(DE3) *E. coli* cells. The protein is obtained as an homogenous polypeptide existing as a mixture of monomer and dimer in a DTT-dependent proportion [83]. These forms contain less than 0.1 iron per β chain. This iron is part of a $(2\text{Fe}-2\text{S})^{2+}$ center as judged from optical absorption and Fe/S colorimetric titrations. The iron sulfur center may be reconstituted under anaerobic incubation with Fe^{2+} and S^{2-} in the presence of DTT. As reconstituted, the β peptide does not take up more than 2Fe and 2S and is fully active for CTP reduction in presence of α_2 . The resulting dark brown protein has an unusual UV-vis spectrum with a broad absorption at around 420 nm and quite a strong band at around 600 nm (Fig. 5). This spectrum is unlike the one obtained from the as-isolated protein. Resonance Raman and Mössbauer spectroscopy indicate that the iron is mainly part of a $(2\text{Fe}-2\text{S})^{2+}$ center [90]. However, at high concentration and/or in the absence of DTT, an other component shows up at the expense of the $(2\text{Fe}-2\text{S})^{2+}$ center. This species is paramagnetic but EPR-silent and has not yet been identified.

Strong chemical reductants such as sodium dithionite (-550 mV) or photoreduced deazaflavin (-650 mV) are necessary to elicit an EPR spectrum of the reduced state [83] (Fig. 6). The signal is rhombic in the presence of glycerol and axial in its absence with g values (2.02, 1.92) characteristic of a $S=1/2$ spin state. At mM concentration additional signals are detected (5.3, 4.7, 2.95, 1.34) tentatively assigned to different $S=3/2$ spin states. The temperature and microwave saturation behavior of the $S=1/2$ signal when compared to known $(2\text{Fe}-2\text{S})^{1+}$ and $(4\text{Fe}-4\text{S})^{1+}$ standard proteins points to the presence of a $S=1/2$ $(4\text{Fe}-4\text{S})^{1+}$ center. The small proportion of higher spin state is in agreement with this conclusion as well as the reactivity of this center toward mild oxidant: with thionin or O_2 , the $(4\text{Fe}-4\text{S})^{1+}$ cluster is primarily oxidised to a mixture of $(3\text{Fe}-4\text{S})^{1+}$ and $(2\text{Fe}-2\text{S})^{2+}$ centers.

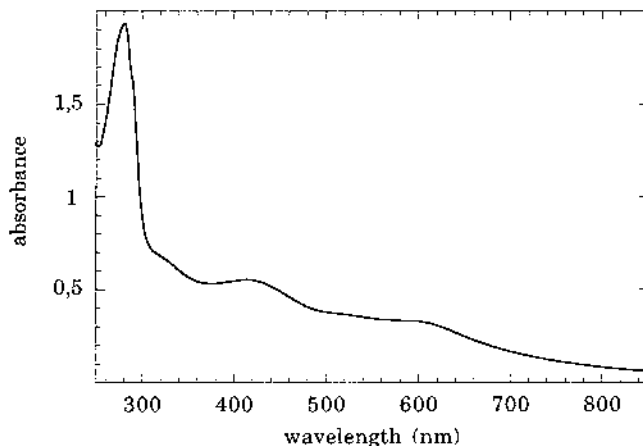


Fig. 5. Electronic spectrum of the small subunit (33 μM in Tris buffer pH 8.0) after reconstitution (2.2 Fe, 2.0 S per β chain).

Recently, an extensive Mössbauer study of this reaction has confirmed that, with a 2-fold molar excess of sodium dithionite, the two $(2\text{Fe}-2\text{S})^{2+}$ present in the reconstituted protein are converted into a mixture of $(4\text{Fe}-4\text{S})^{1+}$, $(4\text{Fe}-4\text{S})^{2+}$ and $(2\text{Fe}-2\text{S})^{1+}$ in a 55/30/15 ratio indicating a 60% efficiency versus the amount of reductant used [90]. This low efficiency may be explained by the finding that, at the high concentration needed for Mössbauer spectroscopy, a fully reduced protein spontaneously reoxidizes in the absence of excess electrons. Such a behavior has one precedent in the iron sulfur protein field: the Fe protein of the multienzymatic complex of nitrogenase from *A. vinelandii* is known to contain a $(4\text{Fe}-4\text{S})$ center linking the two homodimeric peptides of this protein. As for the class III RNR, the $(4\text{Fe}-4\text{S})^{1+}$ is unstable and reverts spontaneously to the $(4\text{Fe}-4\text{S})^{2+}$ form, a

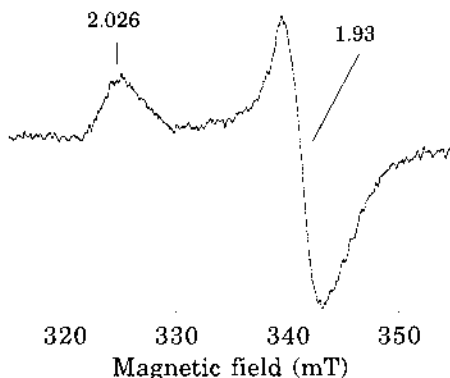


Fig. 6. X-band EPR spectra of the reconstituted small protein (from Ref. [81]).

process which can be slowed down by removing contaminating metal traces from buffers [91].

So, the two $(2\text{Fe}-2\text{S})^{2+}$ centers of class III RNR have the unusual property to undergo a reductive conversion to a $(4\text{Fe}-4\text{S})^{1+}$ cluster, a property which was only very recently observed in 2 other proteins, the biotin synthase [92] and the fnr regulatory protein [93]. As they share with the class III RNR a similar cluster of three cysteines in their sequence it is likely that these three proteins contain related Fe–S centers.

4.3. Class III: the formation of the glycy radical

The formation of the glycy radical is a complex reaction which involves a source of electrons identified in vivo as the ‘flavodoxin system’ [94] comprising NADPH, a reductase (NADPH:flavodoxin oxidoreductase) and flavodoxin, an electron acceptor, *S*-adenosyl methionine (AdoMet), DTT and the Fe–S center of the small subunit. The flavodoxin system can be substituted by strong chemical reductants (dithionite, deazaflavin + light). The deazaflavin reducing system has important technical advantages: first, the overall yield of reduction is near 100% and second, the electron flow stops as the light is turned off. It is then possible to study the one turnover reactivity of the reduced center [85]. The deazaflavin system was used to generate the $(4\text{Fe}-4\text{S})^{1+}$ center of both the β_2 and $\alpha_2\beta_2$ proteins in the absence of DTT. Addition of AdoMet in the dark to the samples induced a high field shift in the EPR *g* values (2.0, 1.91) which was saturable at 1 mM. This was interpreted as the formation of a complex between AdoMet and the proteins. Upon DTT addition to both systems, one could monitor a fast reaction leading to the disappearance of the $S = 1/2$ signal in the case of the β_2 protein and the very fast formation of the glycy radical in the case of the holoenzyme. For both systems these reactions were correlated to the formation of one methionine assayed by HPLC. However, in both cases, two more methionines per protein are formed. These unexpected data suggest that the $(4\text{Fe}-4\text{S})^{1+}$ delivers three electrons at a potential low enough to lead to three successive cleavage reactions.

More recent studies have focused on the physiological ‘flavodoxin’ system as a more realistic electron source for the cleavage reaction. *E. coli* flavodoxin is a 20 kDA monomer which contains one FMN per protein [95]. It is involved in the activation of several enzymes (biotin synthase [96], methionine synthase [97] and most notably pyruvate formate lyase [98]). It is known that with NADPH as the electron donor, the flavodoxin system gives the one-electron reduced EPR active semiquinone form (SQ) as the sole or main product [98,99].

The two redox forms of the protein (hydroquinone, HQ and semiquinone, SQ) have been chemically prepared and assayed for the reduction of the Fe–S center both in β_2 and $\alpha_2\beta_2$ [100]. Upon incubation with the HQ form, two SQs *per* protein are formed. However, as the EPR of the metal center is silent, this suggests that the two $(2\text{Fe}-2\text{S})^{2+}$ of each β monomer have been reduced and that the resulting mixed valent clusters have merged to form an EPR-silent $(4\text{Fe}-4\text{S})^{2+}$ center. Under the same conditions the SQ form is unable to bring about any significant

reduction of β_2 or $\alpha_2\beta_2$. However, upon AdoMet addition to the latter, the characteristic doublet of the Gly $^\circ$ radical could be detected. The yield of Gly $^\circ$ per protein was significantly higher for the SQ system than for HQ (0.6 vs. 0.25, respectively).

These data suggest that the flavodoxin semiquinone is the physiological electron donor of the reaction. More work is necessary to decide what the active redox form of the Fe–S center could be. Nevertheless, it is striking that the reactivity of the system is controlled at least in part by the binding of Adomet.

5. Conclusions

Ribonucleotide reductases are free radical proteins. They have evolved to employ different metallic and organic cofactors according to their availability.

Classes I and III, although showing very different sequences and cofactors, have a strong correspondence as far as their organization and radical generation are concerned. In both cases, a reductive activation takes place, of oxygen for class I and AdoMet for class III. In both cases the resulting species is highly oxidative. In class I the two-electron oxidant ($\text{Fe}^{4+}\text{Fe}^{4+}$) is immediately quenched by an extra reducing equivalent so as to direct the oxidative power to the one-electron oxidation of the tyrosine nearby. In contrast, in class III the activated Adomet generates a one-electron oxidant (Ado $^\circ$) which is used to oxidize a glycine residue. Class II RNR does not hold to this general organization and regulation.

To control the formation of their respective radical, the three classes have evolved quite different mechanisms. In class I, as oxygen is ubiquitous, the reaction is likely controlled at two levels: (1) the formation of the reduced dinuclear center and (2) the radical chain transfer of the oxidative equivalent once the tyrosyl radical is formed. In class II, the formation of the thiyl radical is most probably only controlled by the homolytic cleavage of the Co–C bond. Finally, in class III, the reduction of AdoMet and the generation of the 5'-adenosyl radical is apparently triggered by AdoMet binding most probably at the glycine site. Future work will undoubtedly uncover the detailed mechanisms of these regulation mechanisms.

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