CMLS, Cell. Mol. Life Sci. 54 (1998) 684–695
1420-682X/98/070684-12 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Ribonucleotide reductases and radical reactions

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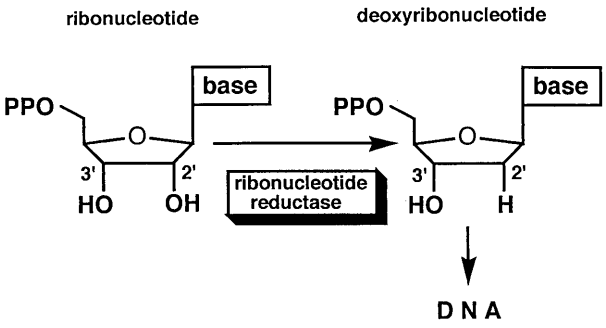
Received 3 December 1997; received after revision 26 February 1998; accepted 27 February 1998

Abstract. Ribonucleotide reductases (RNRs) catalyse the reduction of ribonucleotides to deoxyribonucleotides. They play a pivotal role in the regulation of DNA synthesis and are targets for antiproliferative drugs. Ribonucleotide reductases are unique enzymes in that they all require a protein radical for activity. Class I nonheme iron RNRs (mammals, plants, *Escherichia coli*) use a tyrosyl/cysteinyl radical pair, class II adenosylcobalamin RNRs (prokaryotes, archaea) a cysteinyl radical, class III iron-sulphur RNRs (facultative anaerobes) a glycyl radical. Here we describe the reactivity of these radicals with respect to the natural ribonucleotide substrates as well as to a variety of enzyme inhibitors, radical scavengers, nitric oxide, superoxide radicals and substrate analogues.

Key words. Ribonucleotide reductase; tyrosyl; glycyl; thiyl; radicals; hydroxyurea; nucleoside analogues; nitric oxide; superoxide radical.

Introduction

DNA synthesis depends on a balanced supply of the four deoxyribonucleotides [1]. In all living organisms, with no exception so far, this is achieved by reduction of the corresponding ribonucleotides (scheme 1). At first sight, conversion of a C–OH bond at the 2′-position of the ribose moiety to a C–H bond, in a single-step reaction, seems to be a rather trivial chemical issue. On the contrary, the thermodynamic and the stereoelectronic constraints make it very difficult to explain why there is no precedent for such a reaction in synthetic chemistry [2]. The solution has been found by living cells in the form of a fascinating family of metalloenzymes called ribonucleotide reductases (RNRs) which catalyse the reduction of ribonucleotides to deoxyribonucleotides by NADPH (reduced nicotinamide adenine dinucleotide phosphate) via a complex free-radical chemistry.



Scheme 1.

It is now generally accepted that life was first based on RNA and that the emergence of a ribonucleotide reductase was the key event that allowed the transition from the RNA to the DNA world [3–5]. According to that concept, we would expect to find only one type of

Table 1. Three classes of RNRs.

Class	Source	Structure	Metal site	Radical
I	Ia mammals plants viruses *E. coli* (aerobes)	$\alpha 2\beta 2$	Fe^{3+}–O–Fe^{3+} (nonheme)	tyrosyl/ cysteinyl
	Ib prokaryotes (aerobes)			
II	prokaryotes archaea	α or $\alpha 2$	adenosyl cobalamin (cobalt)	cysteinyl
III	prokaryotes methanogens (anaerobes)	$\alpha 2\beta 2$	[Fe–S] (iron-sulphur)	glycyl

enzyme with the same general structure in all organisms. Instead, in contemporary metabolism we find at least three distinct classes of RNR, which probably are the products of divergent evolution from a common ancestor [3, 4].

In this paper I will not discuss the evolution of ribonucleotide reduction. This aspect was recently presented in review articles by Peter Reichard [3, 4], who has provided the most impressive contribution to this field during the last 40 years. Instead, after a short presentation of the different classes of RNRs, I will discuss the radical chemistry of ribonucleotide reduction, a feature common to all RNRs.

Three classes of RNRs

As shown in table 1, three well-characterized classes of RNRs have been described thus far, with very limited amino acid sequence similarity between them. An exception is the class II enzymes from thermophilic archaea, such as *Pyrococcus furiosus* [5] and *Thermoplasma acidophila* [6], which show significant homology with both class I and class III RNRs present in eukaryotes and bacteria, respectively. This observation suggests that the archaeal enzyme might be the closest possible relative of the primitive RNR [5].

Class I RNRs are strictly aerobic enzymes. They are divided into two subclasses, Ia and Ib. Class Ia RNRs are found in all types of eukaryotes, several viruses, such as the herpes simplex viruses, very few prokaryotes and some bacteriophages. The class Ia prototype is the $\alpha 2\beta 2$ enzyme from *Escherichia coli*, which has been fully characterized. Both the large and the small subunits, called R1 ($\alpha 2$) and R2 ($\beta 2$), have been crystallized, and their three-dimensional stucture determined at high resolution [7–11]. Protein R1 contains the binding sites for both substrates and allosteric effectors. Recently, remarkable structural information about R1 complexes with substrates and effectors was provided by Hans Eklund and co-workers [12]. It shows that the substrate site contains the three conserved redox-active cysteines which were previously shown to participate in ribonucleotide reduction (see below). Protein R2 contains a nonheme di-iron centre, in which the ferric ions are linked by an oxo and a bidentate glutamate bridge on each polypeptide chain. The X-ray structure shows that the metal centre is deeply buried within the protein and that a tyrosine residue (Tyr122) is about 5 Å away from the closest Fe atom. In the active enzyme this tyrosine is in fact a tyrosyl radical, as shown from a variety of spectroscopic studies and from site-directed mutagenesis [13–15]. Protein R2 was the first protein discovered to carry a stable tyrosyl radical, essential for enzyme catalysis.

Class Ib RNRs are also $\alpha 2\beta 2$ enzymes found in bacteria, such as *Lactococcus lactis* [16], *Salmonella typhimurium* [17] and *Mycobacterium tuberculosis* [18], for example. A class Ib enzyme is also found in *E. coli* but is not expressed in sufficient amounts to support growth under normal laboratory conditions [19]. These RNRs are closely related to class Ia enzymes, with similar Fe-radical centres and amino acid sequences except for the lack of the first 50 amino acid N-terminal residues in the large $\alpha 2$ (protein R1) subunit. Also, differences have been observed as far as the allosteric regulation of ribonucleotide reduction is concerned [20]. For example, deoxyadenosine triphosphate (dATP) stimulates reduction of cytidine diphosphate (CDP) under conditions when dATP strongly inhibits all activity of class Ia RNRs. It has also been shown that class Ia RNRs can use thioredoxin or glutaredoxin to shuttle electrons from NADPH to the reductase, whereas class Ib RNRs cannot use thioredoxin. A specific electron transporter, a new redoxin, has been discovered within the class Ib RNR operon [16].

Class II RNRs are found in bacteria and archaea. They generally can work both aerobically and anaerobically and, consequently were found in aerobes, facultative or strict anaerobes. What characterizes a class II RNR is the requirement for adenosylcobalamin as a cofactor. No three-dimensional structure is available so far, but elegant studies from JoAnne Stubbe have demonstrated that, as in class I protein R1, ribonucleotide reduction depends on the presence of three essential redox-active cysteines [21].

Class III RNRs are oxygen-sensitive enzymes found in some facultative anaerobes [4, 22, 23]. The corresponding genes are not expressed under aerobic conditions. On the basis of sequence comparisons, it seems likely that methanogens use a class III enzyme for deoxyribonucleotide synthesis [4]. The prototype is the enzyme

that P. Reichard and myself discovered in 1987 in anaerobically growing *E. coli* cells [22]. Also studied is the enzyme from bacteriophage T4 [23, 24]. This class has not been structurally characterized either. However, biochemical and spectroscopic studies have shown that it is an $\alpha 2\beta 2$ enzyme [25]. The large component $\alpha 2$ contains the substrate and the allosteric effector-binding sites [26]. As there are seven conserved cysteine residues in the α polypeptide [27] and some mutational changes in the phage enzyme led to inactivation (B.-M. Sjöberg, personal communication), it was suggested that ribonucleotide reduction within this class of RNRs also depends on redox-active cysteines. Further studies are needed to confirm this point. A remarkable property of protein $\alpha 2$ is its ability to stabilize a glycyl radical (Gly 680 in *E. coli*) absolutely required for catalysis [24, 28] in the strict absence of molecular oxygen. The small component $\beta 2$ contains one labile $(2Fe-2S)^{2+}$ cluster per polypeptide chain, as shown from its light-absorption, Raman resonance and Mössbauer spectroscopic properties [29].

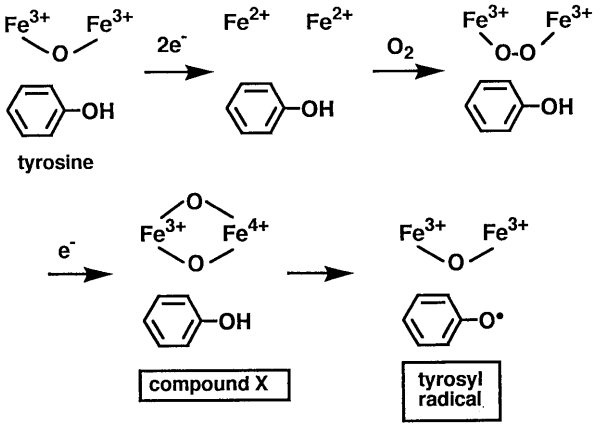
Finally, we should mention the possible existence of a fourth class of RNRs, which uses manganese as the metal cofactor [30]. However, preliminary genetic and biochemical data indicate that the manganese-dependent RNR from *Corynebacterium ammoniagenes* might in fact be a class Ib RNR in which the metal site is occupied by manganese ions (P. Reichard, personal communication). Further work is still needed to confirm the existence of an Mn-RNR.

An interesting observation was that a given organism can have several different RNRs. For example, *E. coli* has a class Ia RNR for aerobic growth but uses a class III RNR for anaerobic growth. Unexpectedly, it also has a class Ib RNR gene whose expression is too low to support growth. There are certainly particular growth conditions, still not identified, under which this enzyme is required. Other combinations have been observed, such as a class Ib and a class III RNR in *Lactococcus lactis* [16] or a class Ia and a class Ib RNR in *S. typhimurium* [17]. There are no examples of organisms carrying a class II RNR together with any other type of RNR.

Mechanism of ribonucleotide reduction

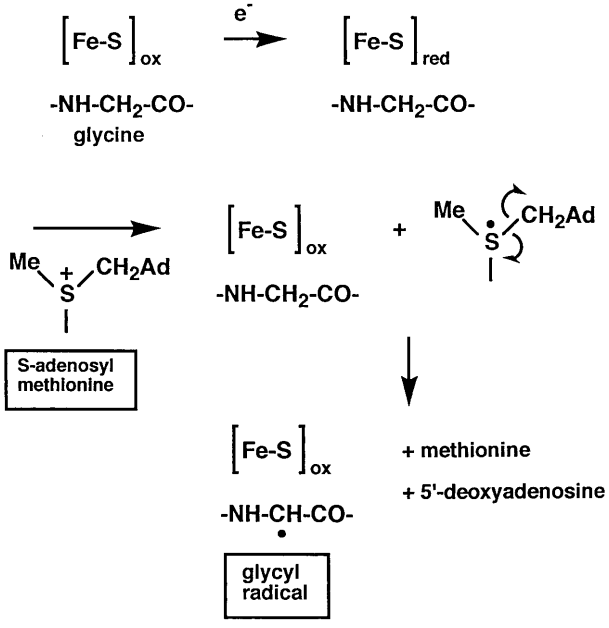
Formation of the essential radical

All ribonucleotide reductases are radical enzymes: they carry a free radical that is essential for catalysis. In class I and class III RNRs, this function resides in a stable tyrosyl and a stable glycyl radical, respectively. A complex chemistry assures the post-translational incorporation of these radicals in the polypeptide chains. This aspect will not be discussed here, as a review article [31]

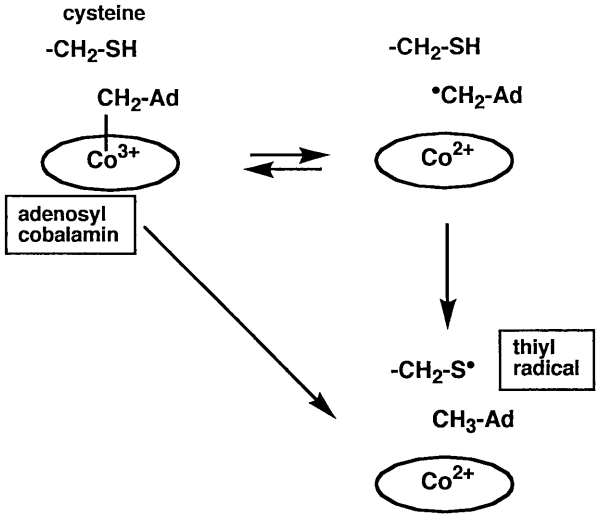


Scheme 2.

and a paper in *J. Biol. Chem.* [32], both published in 1997, treated it in depth. Briefly, it is important to note that in both cases, the metal centre is a key component of the reaction. In class I RNR the nonheme di-iron site catalyses a reductive activation of molecular oxygen to generate a high-valent iron complex, called compound X, which is responsible for the oxidation of a nearby tyrosine residue into a stable tyrosyl radical (scheme 2) [33–38]. In class III RNR the iron-sulphur centre catalyses a reductive activation of *S*-adenosylmethionine to generate a 5′-deoxyadenosyl radical, which is responsible for the hydrogen atom abstraction on a glycine residue (scheme 3) [25, 32, 39–41].



Scheme 3.



Scheme 4.

The situation is less clear in the case of class II RNRs, since no stable radical could be detected. Instead, an organometallic cofactor, adenosylcobalamin (AdoCbl), is required. However, it has been recently shown that during catalysis, protein-bound AdoCbl reacts with a conserved cysteine residue of the active site, leading to an essential transient cysteinyl radical (scheme 4) [42, 43].

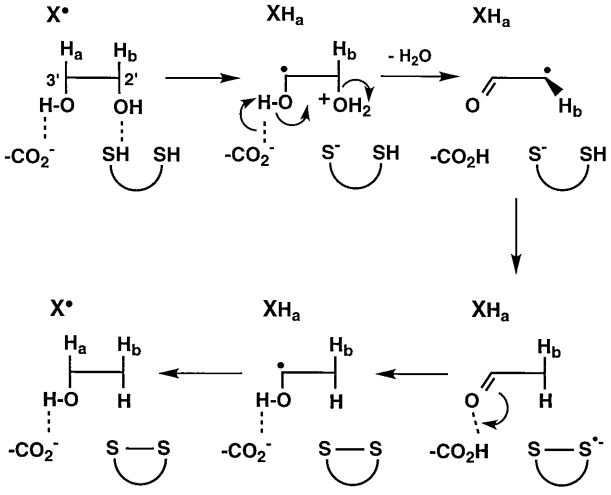


Figure 1. Proposed mechanism for the reduction of ribonucleotides catalysed by ribonucleotide reductase, after J. Stubbe [44].

A radical mechanism for ribonucleotide reduction in class I and II RNRs

The most recent proposal for a radical nucleotide reduction mechanism, common to class I and II ribonucleotide reductases, is shown in figure 1. It is based on elegant investigations, by Stubbe and co-workers, of both *E. coli* and *L. leichmanii* enzymes, using isotopically labelled substrates, mechanism-based inhibitors and site-directed mutants [44].

A free radical on the protein active site, X°, abstracts Ha, the hydrogen atom at the 3′ position of the substrate ribose, to give a 3′ radical. The latter is then activated by hydrogen bonding/deprotonation of the OH group at the 3′ position by a carboxylate (base catalysis) and hydrogen bonding/protonation of the OH group at the 2′ position by a cysteine pair (acid catalysis). The importance of a base such as a carboxylate, while previously anticipated [44], has been fully appreciated only very recently [45, 46]. After loss of H_2O, a new intermediate carbonyl-conjugated radical receives a hydrogen atom from the two cysteines, thus generating a 3′-ketodeoxyribonucleotide. Its reduction by the disulphide radical anion and return of Ha to the 3′ position completes the synthesis of the deoxyribonucleotide with regeneration of the initiating protein-based X° radical and formation of a disulphide. The latter needs to be reduced for the enzyme to be prepared for another turnover.

The reducing equivalents for regeneration of a reduced cysteine pair, in both class I and class II enzymes, are provided by NADPH. Electron transfer chains, such as thioredoxin reductase-thioredoxin in the case of class Ia RNR, are required. However, reduced thioredoxin does not directly inject electrons into the active site. Instead, a second class of cysteine pairs, identified by site-directed mutagenesis as Cys754-Cys759 and Cys731-Cys736 in the C-terminal end of *E. coli* protein R1 and *L. leichmanii* class II RNR, respectively, functions to deliver reducing equivalents from thioredoxin into the active site disulphide, thus regenerating an active enzyme [21, 47, 48].

This mechanism is far from obvious. Its characteristic feature is that the two-electron reduction of a ribo- to a deoxyribonucleotide begins with a one-electron oxidation of the ribose moiety by a radical centre (Ha abstraction) and follows by a three-electron reduction of the intermediate ribose radical. The first radical step is critical, as it is the only way to activate a ribose for reduction. As a matter of fact, SN2 displacement at C-2′ of nucleosides is inhibited by steric and electronic factors. Cation formation (SN1) at C-2′ is precluded by bonding to the adjacent electron-deficient anomeric carbon. Generation of anionic character at C-2′ results in the elimination of the base at C-1′ [49].

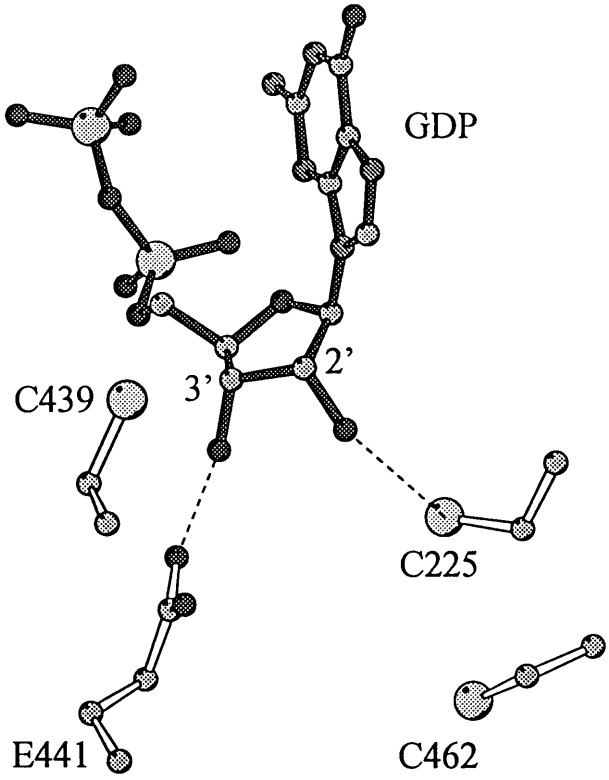


Figure 2. The active site of *E. coli* class I ribonucleotide reductase. The 2′-OH and the 3′-OH are hydrogen-bonded to Cys225 and Glu441, respectively (dotted lines). Cys439 is ideally located for abstraction of the 3′-hydrogen atom [8, 12].

The essential amino acid residues in class I and II RNRs

Site-directed mutagenesis combined with crystallographic studies of both components, protein R1 and protein R2, of class Ia *E. coli* ribonucleotide reductase allow unambiguous identification of the amino acid residues essential for catalysis. The three-dimensional structure of a protein R1-substrate complex gives an exquisite view of the substrate site and the arrangement of important residues around the ribonucleotide [12] (fig. 2). The redox-active cysteine pair is composed of Cys225 and Cys462, present on the α face of the nucleotide. In the initial structure, they formed a disulphide bridge, but by reduction of the crystal with dithiothreitol, the structure of protein R1 in reduced form could be obtained. As expected for redox-active cysteines, the main observed differences in the electronic density between the oxidized and reduced forms were found around Cys225 and, even more pronounced, around Cys 462 [12].

The carboxylate moiety of Glu 441 is in a suitable position in the active site for being the base which deprotonates the 3′-OH group. The key role of that residue for catalysis has been unambiguously shown by site-directed mutagenesis [45]. Furthermore, an interesting recent study with model nucleosides has shown the importance of base catalysis by a carboxylate in the deoxygenation of the 2′ position [46]. In addition, the study also suggested that the same protonated carboxylate would act as an acid in the reduction sequence, since reduction of the 3′-ketodeoxyribonucleotide by the disulphide radical anion is thermodynamically favoured by protonation [46].

The only candidate for the X° radical site is a cysteine residue, Cys439, located on the β face of the ribonucleotide, in agreement with the previous observation that Cys439Ser and Cys439Ala R1 mutants are totally inactive [50]. Even though no cysteinyl radical could be observed during catalysis, it now appears to be the case that Cys439 transiently harbours the radical that abstracts the 3′ hydrogen.

Protein R1 binds the substrates and the allosteric effectors, while protein R2 carries the essential tyrosyl radical inaccessible to solvent and too far away from the substrate to carry out the H-atom abstraction. Consequently, radical chemistry at the substrate site is possible only if there is a mechanism for transferring the radical centre from the tyrosine residue on R2 onto the cysteine 439 residue on R1. It is thus proposed that the function of the tyrosyl radical on R2 is to generate, as soon as the substrate binds to R1, a transient thiyl radical on Cys439 on R1, which then will abstract the 3′ hydrogen of the substrate. This radical transfer is thus probably controlled by substrate and allosteric effector binding. How R1 and R2 communicate is still a matter of investigation.

What is best understood, from the available structural information and from site-directed mutagenesis studies, is the radical transfer pathway (fig. 3). It is likely to consist of a hydrogen-bonding chain of six invariant residues in R2 (Y122, the iron ligands, D84 and H118, and D237, W48, Y356) followed by a hydrogen-bonding chain of three invariant residues in R1 (Y730, Y731 and C439) [9, 10]. A single mutation of one of these residues results in an inactive enzyme [51–53]. It should be noted that only Tyr356 is not visible in the electron density maps, as it belongs to the flexible C-terminus part of R2, shown to be essential for R1-R2 interaction [54]. However, it is possible to build a model for the holoenzyme which locates this part exactly in the cavity beween the Tyr731 region of R1 and the Trp48 region of R2, thus making Tyr356 a good candidate for a radical transfer mediator from Trp48 to Tyr731 [9, 10]. An unusually long distance of about 35 Å separates Tyr122 on protein R2 and Cys439 on protein R1. A long-range electron transfer (ET) mechanism was first proposed for explaining the radical transfer reaction. More recently, it was proposed that the mechanism would be better described as a sequence of hydrogen atom transfers, each one occurring only over very short

distances on the order of 1 Å [9, 10, 55]. The advantage over conventional long-range ET is that charge separation is always kept small. The importance of the hydrogen-bonding network in the radical transfer chain is shown from site-directed mutagenesis. For example, if tyrosines 730 and 731 in protein R1 are changed to phenylalanines, a mutation that destroys the hydrogen-bonding network, the radical transfer is stopped, even though the π ring systems are still there, which might be enough for electron transfer [53]. Though this mechanism is attractive, it should be noted that while several short-lived intermediate amino acid radicals might be expected to be formed, none of them, including the Cys439 radical, has been observed so far.

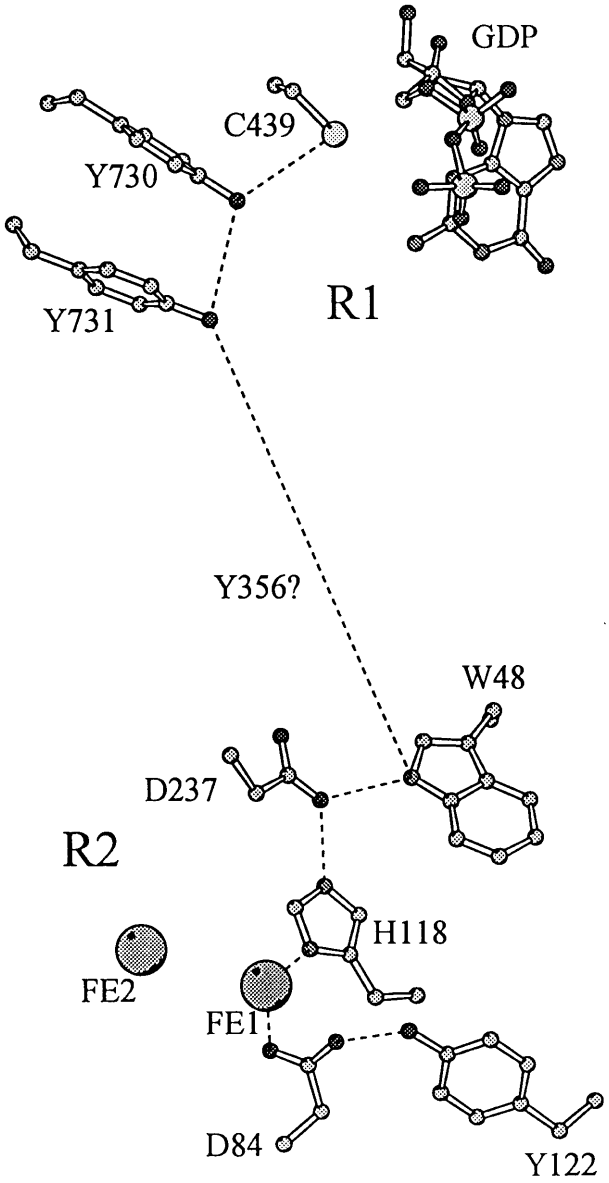


Figure 3. The radical transfer chain in *E. coli* class I ribonucleotide reductase. Tyr122 on protein R2 is connected to Cys439 on protein R1 through the iron ligands and a sequence of hydrogen-bonded amino acid residues, probably including Tyr356 within the disordered R1–R2 interface [9, 10].

Thus far, no three-dimensional structure for a class II RNR has been determined. However, a similar arrangement around the substrate, within the active site, is likely to exist. By comparison of class I *E. coli* and class II *L. leichmanii* RNR sequences and site-directed mutagenesis, the redox-active cysteines of *L. leichmanii* enzyme have been identified as Cys119 and Cys419, and the radical site again as a thiyl radical located on Cys408. In that case, the thiyl radical is not generated by long-range radical transfer [21], as in class I enzymes. Instead, it is produced during oxidation of Cys408 by AdoCbl during a reaction which, surprisingly, does not require substrate binding. It is not clear whether this is a concerted reaction or whether homolytic cleavage of the Co–C bond of protein-bound AdoCbl proceeds before hydrogen-atom abstraction from cysteine by an intermediate adenosyl radical occurs (scheme 4). As a matter of fact, no adenosyl radical could be observed during the reaction. Stubbe and co-workers have shown that a thiyl radical could be trapped by rapid-freeze quench techniques and analysed by electron paramagnetic resonance (EPR) spectroscopy [42, 43]. All the spectroscopic properties are in agreement with a thiyl radical coupled to cob(II)alamin by electron-electron exchange and dipolar interactions.

All the information available so far now shows that even though each *E. coli* class I and *L. leichmanii* class II RNR possesses characteristic primary and quaternary structures and distinct metallocofactors, they proceed by similar mechanisms, involving an essential thiyl radical, represented as X° in figure 1, which initiates the reduction process.

Class III RNRs also proceed via a radical mechanism

Much less is known about the mechanism of ribonucleotide reduction by class III enzymes. However, it is very likely that the reaction proceeds by a radical mechanism similar to that of class I and II enzymes. As a matter of fact, class III enzymes are radical enzymes with glycyl radicals essential for activity [24, 28, 40]. Furthermore, mechanism-based inhibitors of class I and II RNRs are also excellent inhibitors of class III enzymes [56]. Finally, there are a number of conserved cysteine residues within the large subunit, including two CXXC motifs [27]. We thus suspect that the mechanism shown in figure 1 is also valid for class III RNRs. We still do not know whether the glycyl radical is the reagent X° which abstracts the 3′-Ha of the ribose or, instead, oxidizes a cysteine (by direct H abstraction or by long-range radi-

cal transfer) to generate an active thiyl radical, as in class I and II RNRs. The second hypothesis is consistent with the observation that deuterium is partially incorporated in place of the 3′-H within the deoxyribonucleotide product when the reaction is carried out in D_2O [57]. This result indicates that, as in other classes of RNR, the abstracted 3′-Ha is transferred to a site where it is slowly exchanged with the solvent. Cysteine, more than glycine, can be such a site.

Certainly the major difference between class III RNRs and other classes resides in the source of electrons required for ribonucleotide reduction. As mentioned above, in the case of class I and II enzymes, a redoxin, containing a redox-active pair of cysteines, shuttles the electrons from NADPH to a first pair of cysteines at the C-terminus, which then reduces a second pair of cysteines within the active site of the reductase. These cystreines then subsequently provide the hydride equivalent at the 2′ position. Class III RNRs do not utilize a redoxin but formate instead, an excellent two-electron reducing agent with a standard redox potential of −420 mV as the hydrogen donor [58]. During the reaction formate is converted into carbon dioxide. However, we found that hydrogen from formate was totally lost to the solvent during catalysis, indicating that hydrogen is transferred to a site where it exchanges with the protons of water. Cysteine would be a good candidate for such a site, and it is tempting to suggest that also with class III enzymes, the direct hydrogen donor to the substrate is a pair of redox-active cysteines, reducible by formate. This is highly speculative, as there is no chemical precedent for reduction of a disulphide by formate.

Inhibition of ribonucleotide reductase

RNR is a pivotal enzyme for deoxyribonucleotide and DNA synthesis in mammals. Ribonucleotide reduction is the rate-limiting step in the whole process, during the S-phase of the cell cycle, and its complex allosteric regulation provides one of the important mechanisms for controlling the relative intracellular concentrations of the four deoxyribonucleotides [1]. A considerable body of evidence suggests that disturbances in the normal balance is mutagenic and may lead to other genetic abnormalities.

Furthermore, an excellent correlation between RNR activity and the rate of proliferation of a given tissue has been observed [59, 60]. High activity is present in regenerating liver, embryonic tissues, thymus and so on. Tumour cells or virus-infected cells also have much higher RNR activity than the corresponding wild-type cell lines [61].

Consequently, as inhibition of RNR generally results in inhibition of DNA synthesis and cell proliferation, this enzyme is considered to be one of the important targets for anticancer and antiviral drugs. At first sight, it seems rather surprising that so few inhibitors of RNR are used in clinics as drugs, in comparison to the huge number of compounds which have been tested all over the world during the last 30 years. In fact the importance of the enzyme for both cancer and normal cells is a major drawback for this strategy, and in general RNR inhibitors have serious secondary toxic effects owing to their lack of selectivity.

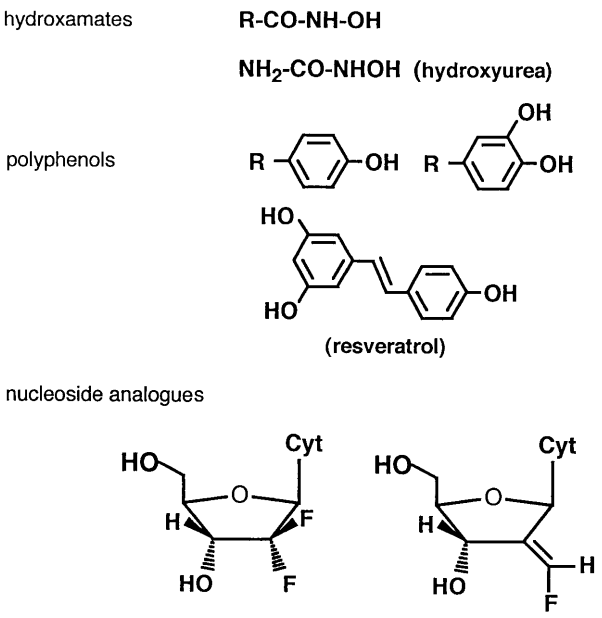


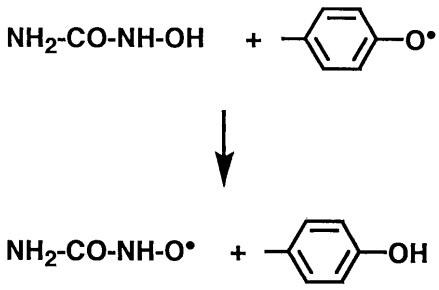
Figure 4. Inhibitors of ribonucleotide reductases.

There are, however, conditions under which this selectivity can be improved. For example, treatment of herpes simplex could be achieved with RNR inhibitors, as the virus expresses its own RNR during infection. The structural and functional differences between the viral and the cellular enzymes are significant and make possible selective destruction of the infected cells [62].

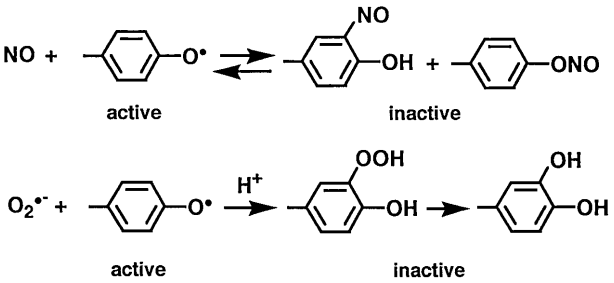
The radical nature of RNR and consequently the involvement during catalysis of key free-radical intermediates, either protein- or substrate-bound, are the specific characteristics of this family of enzymes. But because relatively few enzymes share this property, these radicals are attractive targets for selective inhibition of RNR and radical scavengers turn out to be efficient inhibitors.

Radical scavengers

Examples of hydroxamic acids and polyphenols are shown in figure 4 [63–67]. They all react by providing a hydrogen atom directly to the small R2 protein, during a reaction which does not require but is significantly



Scheme 5.



Scheme 6.

affected by protein R1, and converting it into inactive forms, namely metR2, in which the iron centre is still present but the tyrosyl radical is lacking, and apoR2, in which both are absent [68–70]. In this reaction the drug is converted into a radical (scheme 5).

What is still unclear is whether these compounds penetrate into the protein to react with the tyrosyl radical directly or whether they proceed at the surface of the protein via long-range hydrogen atom transfer, as the refined structure does not show any obvious access routes from the surface to the buried radical.

Hydroxyurea is the most well-known inhibitor of RNR. It is used in clinics as an anticancer agent. Recently it was revisited in the context of the search for new drugs against human immunodeficiency virus (HIV), since a combination of hydroxyurea and (zidorridine) AZT or ddI (didanosine) proved to have synergistic antiviral effects [71–73]. It is conceivable that inhibition of RNR potentiates the incorporation of these nucleoside analogues into elongating DNA by depleting the natural deoxyribonucleotide pools [74, 75]. Other mechanisms may explain this synergy, for example the activation of nucleoside salvage pathways which would be responsible for an improved cellular uptake of the nucleoside analogues [73].

Unfortunately, hydroxyurea does only a reversible inhibition, since cells contain activities which efficiently reincorporate the tyrosyl radical into protein R2 [35, 36]. Furthermore, cells rapidly develop resistances to the drug. High doses, which eventually become toxic, are thus required. There is still a need for less toxic and more efficient inhibitors of RNR. We recently found that resveratrol, a natural phytoalexin found in grapes, was such an inhibitor and might be an attractive compound to investigate as an anticancer agent in humans [76].

Nitric oxide and superoxide radical

It is interesting to mention two other tyrosyl radical scavengers which have very little future as drugs but may have important physiological roles.

The first one is nitric oxide (NO). Among a number of biological functions, it acts as a mediator of the cytotoxic effects of activated macrophages [77]. It has been shown to be responsible for the profound inhibition of DNA synthesis in target tumour cells, bacteria, intracellular parasites or viruses, thus contributing to the host immune defence against rapidly proliferating pathogens.

There is now ample evidence that RNR is one of the key targets of NO [78]. This is not surprising, since NO carries an unpaired electron and is a paramagnetic molecule. We have shown that NO reacts with the tyrosyl radical of R2 very efficiently through a rather unique radical-radical coupling reaction (scheme 6) [79]. While it was the first time that such a coupling reaction was shown for the tyrosyl radical of RNR, few examples in which NO couples to a free radical were known. One is the reaction with the superoxide radical, giving rise to the peroxynitrite anion.

An unexpected observation was that tyrosine-NO adducts have the ability to slowly dissociate back to the tyrosyl radical, when NO progressively disappears from the solution. This seems to be a general property of the reaction of NO and phenoxyl radicals, as shown from model chemical studies [80]. Thus the coupling reaction is reversible, and the equilibrium between the active and inactive forms of protein R2 is finely controlled by the concentration of NO. It is tempting to suggest that NO might finely tune RNR activity through its unique reactivity with the tyrosyl radical of R2 [79].

A second example is the superoxide radical. RNR is sensitive to oxidative stress conditions and needs to be protected by superoxide dismutase and catalase [81, 82]. We have shown that superoxide radicals efficiently destroy the enzyme tyrosyl radical [83]. This is an interesting observation, since there are exceedingly few examples to date of direct reactions of superoxide with a biological target which would explain its toxicity and the need for superoxide dismutase in all aerobic organisms. In contrast with the reaction with NO, superoxide-dependent inactivation of R2 is irreversible. We have proposed that the reaction proceeds by a radical-

radical coupling between a tyrosyl radical and superoxide, which gives rise to irreversibly oxidized forms of tyrosine, tyrosine peroxide and then 3,4-dihydroxyphenylalanine (scheme 6) [83].

Nucleoside analogues: mechanism-based inhibitors

The inhibition strategy described above is based on the scavenging of the R2-bound radical. However, as discussed previously, transient free radicals are also generated within protein R1 during catalysis initiated by substrate binding. There is thus also a possibility of inhibiting RNR with the help of nucleotide analogues which would bind to protein R1 and trap these intermediates. 2′-substituted 2′-deoxyribonucleotide derivatives (fig. 4) have been shown to be very potent mechanism based inactivators of RNR [44, 84–87]. Among them, some, like 2′,2′-difluorodeoxycytidine (gemcitabine) and 2′-methylenefluoro-2′-deoxycytidine are in clinical trials for their potent chemotherapeutic efficacy against leukaemias and solid tumours [88]. In the latter case, the modification at the 2′ position stabilized a transient nucleotide-based radical during inactivation [85].

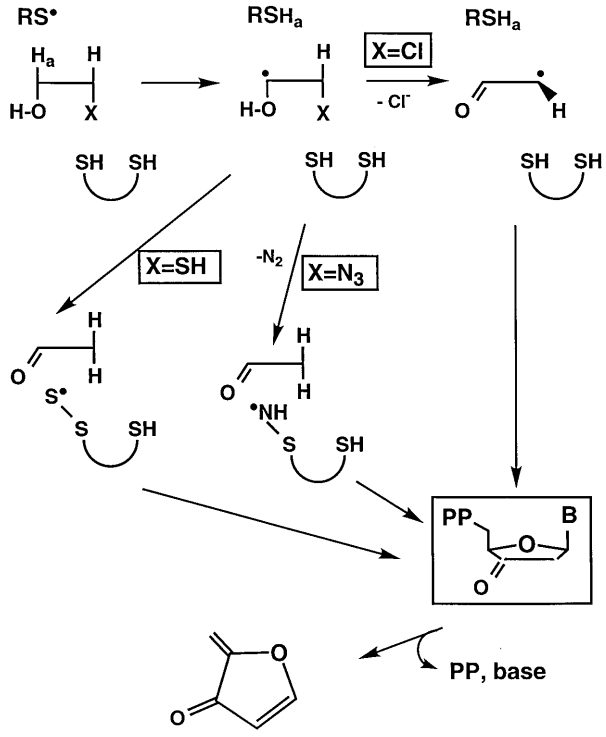


Figure 5. Nucleoside analogues as mechanism-based inhibitors of ribonucleotide reductase. Different pathways leading to secondary protein radicals and to the toxic 2(*H*)-methylene-3-furanone [44, 86].

A summarized view of the mechanism responsible for enzyme inactivation by 2′-X-nucleotide analogues, following Stubbe's proposal [44], is shown in figure 5. The initial step, as in the case of natural substrates, is radical transfer from the R2 tyrosyl radical to generate a thiyl radical which abstracts the 3′ hydrogen atom Ha. Two possibilities then exist for the elimination of X.

In the first case (with X = Cl, for example), loss of X in the anionic form, concommitant with deprotonation of the 3′-OH, results in formation of a 2′-deoxy-3′-ketonucleotide radical. In this step, in contrast with the normal reduction process, the cysteine pair on R1 is not involved. The difference in its protonation state, at the end of this step, is supposed to be responsible for the subsequent inactivation process. Return of Ha to 2′-C to give the 3′-ketodeoxynucleotide can occur either with regeneration of the thiyl radical or from the cysteine pair with loss of the thiyl radical (and consequently loss of the primary initiating tyrosyl radical). Dissociation of the ketone from the enzyme and successive β-eliminations generate the electrophilic 2(*H*)-methylene-3-furanone, which inactivates protein R1 by Michael addition/alkylation.

In the second case (X = N3 or SH), loss of X in the radical form results in the formation of a secondary protein-bound radical, stable enough to be trapped and observed by EPR spectroscopy, together with the 3′-ketodeoxynucleotide [86, 87, 89]. The latter may then be converted to the toxic furanone as described for X = Cl. In that case the tyrosyl radical is totally lost and R2 inactivated, since the initial thiyl radical is not regenerated. As a matter of fact, the new free radicals, identified as Cys–N°–H (X = N3) and perthiyl Cys–S–S° (X = SH) radicals, are now located on the cysteine pair of the active site [87, 88, 90]. It is thus remarkable that the nucleotide analogues have allowed a radical transfer from the cysteine at the β face of the substrate to the cysteine pair on the α face, thus further prolonging the radical transfer pathway within protein R1.

Conclusion

Free radicals are believed to be highly unstable, reactive and toxic species. For that reason, until recently it was hardly conceivable that they might serve as intermediates during selective and regulated biological reactions. This is true as long as radicals are generated in test tubes where their instability is due to very efficient radical-radical couplings (dimerization) and abstraction of H atoms from solvent molecules, for example.

However, if they are formed in sterically and electronically constrained environments, within a protein for example, they can be greatly stabilized. In RNRs, the

tyrosyl (class I) and the glycyl (class III) radicals are generated in such a site, with no abstractable H atom, and are protected by the whole polypeptide chain from dimerization reactions [90, 91].

Furthermore, what we can learn from RNRs is that free radicals can be controlled to achieve very selective and efficient reactions. It is remarkable that DNA synthesis, such an important biological event for life, is under the control of radical chemistry. In fact, once mechanisms for fine-tuning the reactivity of a radical centre have been set up, this is a very efficient way to store one oxidizing equivalent and use it for H-atom abstractions, such as the one required for initiating ribonucleotide reduction [92]. More examples will certainly show the importance of controlled protein-bound free radicals in biological catalysis in the future [93].

Acknowledgements. I thank M. Eriksson (Uppsala, Sweden) for providing figures 2 and 3.

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