Structure and Reactivity of the Metal Centers of Ribonucleotide Reductases

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The activation of the three classes of ribonucleotide reductases as free radical enzymes is reviewed. Class I uses O_2 and a diferric μ-oxo center to generate a stable tyrosyl protein radical. Class II operates with adenosyl cobalamin as the precursor of a putative transient thiyl protein radical. Class III forms an O_2-sensitive protein glycyl radical by the concerted action of an iron–sulfur cluster and (*S*)-adenosyl methionine.

Introduction: The Three Classes of Ribonucleotide Reductases

DNA replication requires a supply of building blocks, the four deoxyribonucleotides (dNTPs). These are produced by reduction of the corresponding ribonucleotides[1]. In all living organisms, this reaction, catalyzed by the allosterically regulated enzyme ribonucleotide reductase, is the only one that provides new dNTPs for DNA replication and repair[1]. Three different classes of enzymes are known, each with a distinct protein structure and a specific metal center but all requiring a protein radical for catalysis[2] (Figure 1).

Class I reductases are aerobic enzymes present in all higher organisms and certain microorganisms. They consist of two homodimers named R1 and R2, both recently structurally characterized[3,4]. Protein R1 contains the binding sites for both substrates and allosteric effectors, and redox active cysteines for the reduction of ribonucleotides. Electrons are provided by NADPH, through electron transfer chains (thioredoxin or thioredoxin reductase for example). Protein R2 contains a non-heme diiron center in which the

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Etienne Mulliez (left) was born in Paris in 1949. He obtained his Ph. D from the Université René Descartes (Paris V) in 1979, under the direction of Prof. J. C. Chottard. He then undertook a postdoctoral fellowship at the Pennsylvania State University, USA, where he worked on the myo-inositol dioxygenase multienymatic complex in the group of Prof. G. A. Hamilton. In 1982 he became a Research Associate of the CNRS at the Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (Paris V) working on chemical models and biochemical studies of the non-heme iron active site of lipoxygenases. Since 1991 he has been a Research Associate in the group of Marc Fontecave, working on biochemical studies of class III ribonucleotide reductases.

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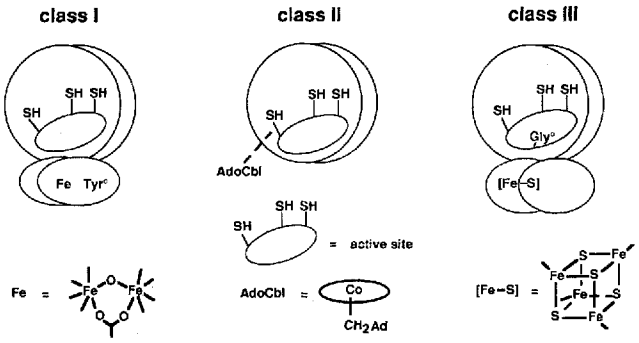
Figure 1. The three classes of ribonucleotide reductases



two ferric ions are μ-oxo bridged, and an adjacent tyrosyl radical[5,6]. It is proposed that the function of the radical is to abstract, through a long-range electron transfer pathway, one electron from a cysteine residue of R1 and thus to generate a thiyl radical in the proximity of the substrate[6-7].

Class II enzymes are found in many microorganisms, both aerobes and anaerobes. They consist of a single polypeptide, either as a monomer or as a dimer, and use adenosylcobalamin (AdoCbl) to generate a cysteinyl radical[8]. As in class I enzymes the substrates are reduced by redox active cysteines and electrons are provided by enzymatically reduced thioredoxin or glutaredoxin.

Class III enzymes are found in some anaerobically growing facultative anaerobes and are extremely sensitive to oxygen. The enzyme from *Escherichia coli* is an $\alpha_2\beta_2$ tetramer[9]. The large subunit α_2 (2×80 kDa) contains binding sites specific for allosteric effectors (nucleoside triphosphates) that regulate the activity and specificity of the enzyme[10]. It is likely that it also contains the binding site for the substrates because it harbors, in its active form, a glycyl radical absolutely required for activity[11]. Whether this radical serves to generate a thiyl radical as in class I and II enzymes is still speculative. The small subunit β_2 (2×17.5 kDa) contains an iron–sulfur center. Based on EPR experiments, preliminary results have suggested that this center is a [4Fe–4S] cluster which brings the two β chains together, but more work is necessary to confirm this point[9]. The radical enzyme is competent for the reduction of ribonucleotides, at the expense of the reducing equivalents of formate, as shown by the stoichiometric production of CO_2 during turnover[12]. So, at variance with the two other classes of RNRs, the class III enzyme employs a low molecular weight compound as the external reductant.

It is clear that, in all classes, the post-translational activation of the enzyme consists of the specific generation of a protein radical. This radical serves for the abstraction of the hydrogen atom at the 3′ position of the ribose moiety, which is a prerequisite for ribonucleotide reduction[13]. In this paper, we describe the chemical mechanisms of the activation reactions, with special emphasis on the role of the metal centers.

Activation of Class I Ribonucleotide Reductases

The function of the iron center is in the formation of the tyrosyl radical cofactor. This reactions is O_2-dependent and occurs spontaneously upon addition of Fe(II) and O_2 to the inactive apoprotein, which lacks both the iron and the radical[14]. MetR2, which contains a diferric center but no radical, can also be activated under aerobic conditions. We have shown that the reaction requires a reducing system (an NADPH: flavin oxidoreductase in *E. coli*) that delivers electrons to the iron center[15]. It is thus clear that in all cases reduced R2, the diiron(II) form, is the key intermediate. During reaction with O_2, both the tyrosyl radical and the diferric center are generated:

$$\text{R2-[Fe(II), Fe(III)]} + O_2 + e^- + \text{TyrOH} + H^+ \rightarrow \text{R2-[Fe(III)–O–Fe(III)]} + H_2O + \text{TyrO}^\bullet$$

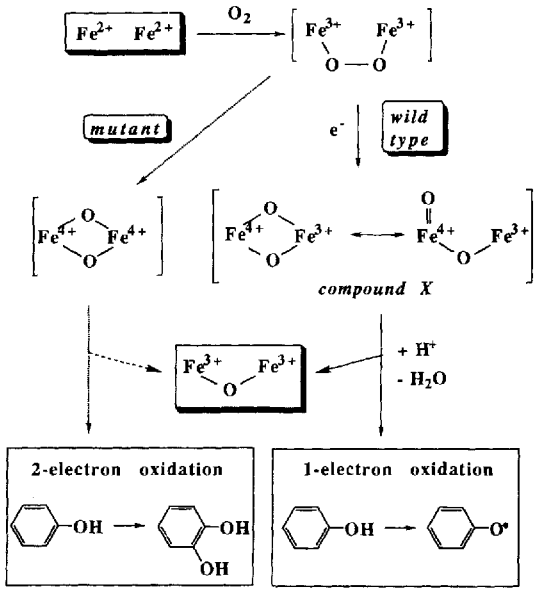
As shown in the equation, one extra electron is required for the four-electron reduction of O_2. There is now accumulating evidence that this is provided by one ferrous ion[13,16].

Recently the reaction of O_2 with the diiron(II) center of RNR has been studied by rapid freeze-quench spectroscopic methods (EPR, ENDOR and Mössbauer)[13,17,18,19]. One intermediate, named compound **X**, was detected and characterized. Compound **X**, an $S = 1/2$ species, contains one oxidizing equivalent above the diferric state and is catalytically competent for oxidizing the tyrosine residue to its essential radical state. It was first described as a coupled system consisting of two high-spin Fe(III) centers plus a ligand radical[13,17]. However, recent reanalysis of the ENDOR and Mössbauer results now strongly suggests that compound **X** carries a spin-coupled Fe(III)Fe(IV) diiron center[19]. Furthermore, ^{17}O ENDOR experiments have shown that both atoms of the reactant O_2 remain bound to iron within compound **X**, in agreement with the observation by resonance Raman spectroscopy that the final oxo bridge of the diferric cluster is derived from O_2[20]. An $S = 1/2$ model Fe(III)Fe(IV) complex containing an $Fe_2(\mu\text{-}O)_2$ core was obtained during reaction of H_2O_2 with a (μ-oxo)diiron(III)[6-Me-TPA=*N*-(6-methyl-2-pyridylmethyl)-*N*,*N*-bis(2-pyridylmethyl)amine] complex[21]. Its EPR properties are strikingly similar to those of compound **X**.

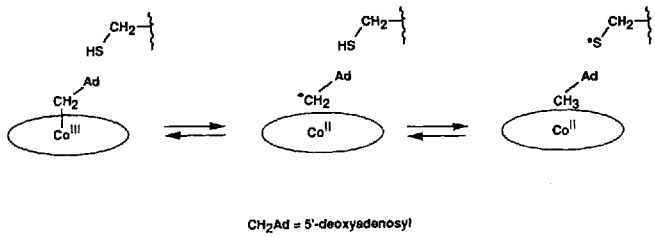
Although no other intermediates could be detected it seems very likely that compound **X** derives from the one-electron reduction of a diferric-peroxo species, similar to compound **L** formed during addition of O_2 to the diiron(II) center of methane monooxygenase[22]. This assumption is supported by the observation that the tyrosyl radical can be produced during reaction of the diferric center of metR2 with H_2O_2[23,24].

All these results support the mechanism shown in Scheme 1. We have completed the scheme by introducing an Fe(IV)Fe(IV) species which, under certain circumstances (mutation of a phenylalanine function, adjacent to the iron center, to tyrosine), may accumulate and be responsible for two-electron, and not one-electron, oxidations (i.e. transformation of the introduced tyrosine into dopa)[25].

Scheme 1



Scheme 2



Activation of Class II Ribonucleotide Reductases

The diverse molecular functions of AdoCbl can be summarized in terms of one generalized function, which consists of the coenzyme structure serving as a molecular reservoir for the reversible dissociative generation of an alkyl radical (the 5′-deoxyadenosyl radical)[26]. The cobalt–carbon bond (which has a dissociation enthalpy of approximately 31 kcal mol^{-1} in ethylene glycol) is sufficiently strong for the coenzyme to be intrinsically stable but, at the same time, sufficiently weak that an acceleration of the dissociation by several orders of magnitude is possible by association with AdoCbl enzymes.

In the case of class II RNRs, AdoCbl also serves as a radical generator. However, thus far an intermediate 5′-deoxyadenosyl radical has not been observed, even though stopped-flow UV-vis and Rapid Freeze-Quench EPR spectroscopies revealed that cob(II)alamin was formed[8]. The axial ligand of AdoCbl was recovered as 5′-deoxyadenosine, with a rate constant identical to that observed for formation of cob(II)alamin.

Recently, an EPR-active intermediate species was trapped by rapid freeze-quench techniques[8]. Again, this intermediate was produced with the same rate constant as cob(II)alamin. When the enzyme was labeled with deuterated cysteines the EPR spectrum of the intermediate exhibited narrowed hyperfine features relative to the corresponding unlabeled enzyme and was thus interpreted to represent a thiyl radical coupled to a Co(II) center.

Furthermore, recent site-directed mutagenesis studies have shown that Cys408 of *Lactobacillus leichmanii* class II RNR is necessary for nucleotide reduction and supported the notion that this is the protein radical responsible for initiating the catalysis[27].

As shown in Scheme 2, class II RNRs utilize AdoCbl for generating 5′-deoxyadenosine, cob(II)alamin and a cysteinyl radical that is responsible for the abstraction of the hydrogen atom at the 3′ position of the ribose moiety.

Activation of Class III Ribonucleotide Reductases

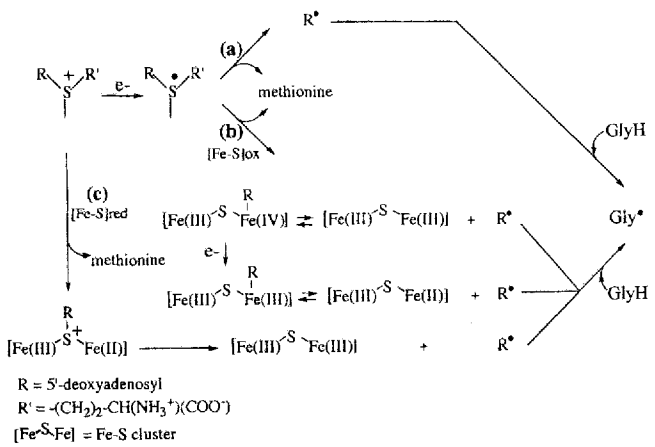
After purification by affinity chromatography on dATP sepharose, the isolated enzyme is inactive. During anaerobic incubation with (*S*)-adenosylmethionine (AdoMet), in the presence of dithiothreitol, KCl and a reducing enzyme system comprising flavodoxin, $NADP^+$: flavodoxin reductase[28], the glycyl radical is generated on the large protein and the catalytic activity restored[11,29]. In the process of the activation, stoichiometric amounts of methionine and 5′-deoxyadenosine are formed[30]. This suggests that AdoMet is the site of a redox reaction.

Very little is known about the redox chemistry of acyclic sulfonium groups[31]. The reduction of sulfonium compounds has mainly been investigated by electrochemistry. At low potential (between −2 and −1 V) an irreversible reduction is observed. In some cases the resulting sulfuranyl radical has been characterized at low temperature[32] but, in general, a very fast cleavage reaction is observed, the most stable alkyl radical being formed. In the case of aryl dialkyl sulfonium compounds, two mechanisms have been proposed. The reaction proceeds either via a π-anion radical sulfonium intermediate or by a concerted or nearly concerted mechanism in which bond breaking is concomitant with electron acceptance. Recent reexamination of the electrocleavage reaction has led to the proposal that the two main factors governing this type of mechanism are the LUMO energy and the bond strength of the starting molecule[33]. The higher the former and the weaker the latter, the greater the tendency for a concerted mechanism to prevail over a stepwise mechanism and vice versa.

Few chemical reductants have been tested for their ability to reduce sulfonium groups. The Japanese group of Hori has shown that Mg(0), Zn(0), and especially samarium diiodide are good reductants of sulfonium compounds and are of preparative value[34]. Nothing is known about the mechanism of these reactions.

The activation of the anaerobic ribonucleotide reductase is being investigated in our laboratory and preliminry results allow the proposal of a reaction mechanism. Using commercially available [*methyl*-^{3}H]-AdoMet, at high specific activity, it is possible to show that the small protein of the reductase is able to catalyze the cleavage of AdoMet into [*methyl*-^{3}H]methioine and 5′-deoxyadenosine[35]. This reaction does not require the presence of the large protein. As the apo form

Scheme 3



is devoid of this activity, it is clear that the Fe–S center is directly involved in the reaction. In addition, the efficiency of the reaction is directly correlated to the reducing power of the electron-donating system. When the latter is the physiological flavodoxin system the reaction stops before one turnover has been completed. In the presence of a potent reducing agent (photoreduced 5′-deazaflavin $E_0' = -0.65$ V), one mole of methionine is formed per mole of β_2. Additional evidence points to an irreversible inactivation of the protein, presumably from a covalent reaction of the derived radical. On the other hand, the complete system ($\alpha_2\beta_2$) makes exactly one cycle in the presence of the flavodoxin system, whereas 5–10 cycles are completed with reduced 5′-deazaflavin. In the latter case, the fast reduction of the glycyl radical might compete with the inactivation reaction.

Also, for the β_2-catalyzed reductive cleavage of AdoMet, it has been shown that DTT acts as a very potent stimulator of the reaction but that its presence is not absolutely required for methionine production. More work is necessary to unravel the molecular basis of this effect. Based on the current data the mechanisms shown in Scheme 3 may be put forward:

In pathway (a), it is proposed that a 5′-deoxyadenosyl radical is generated by homolytic cleavage of the intermediate sulfuranyl radical, the latter coming from one-electron reduction of AdoMet by dehydroflavodoxin (which might be assisted by the metal center).

In pathway (b), the cluster gives one electron to the sulfuranyl radical and can exchange a labile ligand (either carboxylate, histidine or water) to generate either a Fe(IV)-5′-deoxyadenosyl or, if reduced, a Fe(III)-5′-deoxyadenosyl species, these species being in equilibrium with the free 5′-deoxyadenosyl radical and the reduced clusters. This chemistry is much like that carried out by the AdoCbl cofactor and has precedent in the field of mononuclear iron complexes[37]. Whether it is operative with iron–sulfur clusters remains to be demonstrated.

In pathway (c), there is no need for a labile ligand on the Fe–S cluster. The increased nucleophilicity of the bridging sulfide makes it competent for a nucleophilic substitution on the 5′-deoxyadenosyl moiety of AdoMet[36]. Homolytic cleavage of the bridging sulfonium releasing the 5′-deoxyadenosyl radical would then be facilitated by the reduced nearby metal.

AdoMet is known to be a potent and highly versatile methylating agent yielding (*S*)-adenosyl homocysteine as the coproduct. Its function as a source of the 5′-deoxyadenosyl radical described here is rather unusual. However, other biological systems are known to use AdoMet for a similar function. For example, a putative 5′-deoxyadenosyl radical has been suggested in the cases of the pyruvate formate lyase-activating enzyme[38], lysine 2,3 aminomutase[39] and biotin synthase[40]. The latter two contain $[4Fe-4S]^{3+}$ and $[2Fe-2S]^{2+}$ clusters respectively. The former is iron-dependent but no iron–sulfur cluster could be detected although the sequence of the protein shows strong homologies with that of the small protein of the anaerobic ribonucleotide reductase.

Thus, the reductive cleavage of AdoMet seems to be a metal-dependent process and future work will focus on the understanding of this reaction.

Conclusion

It is striking that all ribonucleotide reductases known so far employ radical mechanisms. Protein radicals are generated as a prerequisite for ribonucleotide activation and reduction. It is also striking that, in all cases, the generation of these radicals is under the control of a metal cofactor. In this review article, we have stressed the specific role of these cofactors during the formation of the radicals. However, they may have other functions (e.g. protein structure, stabilization of the radical, and modulation of their reactivity) which have not yet been studied. Ribonucleotide reductases thus belong to a new class of enzymes, named free-radical enzymes, which use both metal ions and radicals for their activity[41,42].

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