Ribonucleotide reductase metallocofactor: assembly, maintenance and inhibition

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Abstract

Ribonucleotide reductase (RNR) supplies cellular deoxyribonucleotide triphosphates (dNTP) pools by converting ribonucleotides to the corresponding deoxy forms using radical-based chemistry. Eukaryotic RNR comprises α and β subunits: α contains the catalytic and allosteric sites; β houses a diferric-tyrosyl radical cofactor (Fe^{III}_2-Y•) that is required to initiates nucleotide reduction in α. Cells have evolved multi-layered mechanisms to regulate RNR level and activity in order to maintain the adequate sizes and ratios of their dNTP pools to ensure high-fidelity DNA replication and repair. The central role of RNR in nucleotide metabolism also makes it a proven target of chemotherapeutics. In this review, we discuss recent progress in understanding the function and regulation of eukaryotic RNRs, with a focus on studies revealing the cellular machineries involved in RNR metallocofactor biosynthesis and its implication in RNR-targeting therapeutics.

Keywords

ribonucleotide reductase (RNR); diferric-tyrosyl radical (Fe^{III}_2-Y•); iron homeostasis

Introduction: RNR enzymes

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleoside 5′-di- or triphosphates (NDPs or NTPs) to deoxyribonucleoside 5′-di- or triphosphates (dNDPs or dNTPs), the only pathway for *de novo* synthesis of the monomeric building blocks for both DNA replication and DNA repair (Reichard, 1993; Stubbe et al., 2001). All cellular organisms that synthesize their own DNA require RNR. Some viruses even carry their own copy of RNR in their genomes, likely to facilitate viral replication in the infected host cells (Boehmer and Lehman, 1997; Kurita and Nakajima, 2012). All RNRs share a common catalytic mechanism in which a metal co factor is involved in oxidation of a conserved cysteine within the active site to a thiyl radical to initiate nucleotide reduction (Stubbe and van Der Donk, 1998; Hofer et al., 2012).

RNRs are grouped into three main classes based on their different metallocofactor requirements. Class I RNRs are oxygen-dependent enzymes that occur in both eukaryotes and eubacteria and contain a dinuclear metal cluster (Fe or Mn) (Bollinger et al., 2008; Cotruvo and Stubbe, 2011). Class II RNRs, found in both aerobic and anaerobic microbes, are oxygen-independent and use a cobalt-containing cobalamin (vitamin B_{12}) cofactor (Eklund et al., 2001; Kolberg et al., 2004). Class III RNRs, found in anaerobic microorganisms, are oxygen-sensitive anduse a $[4Fe\text{-}4S]^{1\ +/2+}$ cluster coupled to *S*-adenosylmethionine (SAM) for catalytic activity (Fontecave et al., 2002; Logan et al., 2003).

Class I RNRs are further grouped into three subclasses according to their metal cluster identities. All class I RNRs share the same structural fold consisting of two subunits, α and β. α binds the substrates NDPs/NTPs, while β houses the dinuclear metal cluster required for catalysis (Reichard, 1993; Mowa et al., 2009; Cotruvo and Stubbe, 2011). In class Ia RNRs it is a diferric-tyrosyl radical (Fe^{III}_2-Y•) (Sjöberg and Reichard, 1977), in class Ib a dimanganese-tyrosylradical (Mn^{III}_2-Y•) (Willing et al., 1988; Cotruvo and Stubbe, 2010), and in class Ic a proposed $Mn^{IV}Fe^{III}$ cofactor (Jiang et al., 2007). While all three subclasses of class I RNRs occur in prokaryotes, most eukaryotic genomes from yeast to human only encode class Ia RNRs (Nordlund and Reichard, 2006; Cotruvo and Stubbe, 2011). This review will discuss recent studies in understanding the mechanisms underlying regulation of the eukaryotic class Ia RNRs, especially the biosynthesis of the metallocofactor in β.

The active form of class Ia RNR holoenzymes has been proposed to have a quaternary structure of $(α_2)_n(β_2)_m$ (*n* = 2, 4, 6, *m* = 1 or 3, depending on the organisms) (Kashlan and Cooperman, 2003; Rofougaran et al., 2006; Wang et al., 2007; Aye et al., 2012a; Minnihan et al., 2013a). In addition to the active site, α also contains allosteric sites for controlling overall activity and substrate specificity (Jordan and Reichard, 1998; Eklund et al., 2001). During each turnover cycle, a transient thiyl radical is generated within the active site of α by oxidation of a conserved cysteine residue. This oxidation occurs by the Y• in β over a long distance (~35Å), via a specific proton-coupled election transfer pathway involving conserved aromatic residues in both α and β subunits (Reece et al., 2006; Minnihan et al., 2013b).

Although the prototype class Ia RNR comprises homo-dimeric $α_2$ and $β_2$ subunits, some eukaryotic genomes encode multiple α and β isoforms, adding another layer of complexity for configurations of the RNR holoenzyme. The human genome encodes one a (RRM1) and two βs (RRM2 and RRM2B or p53R2) (Tanaka et al., 2000). The budding yeast *Saccharomyces cerevisiae* has two α isoforms (encoded by *RNR1* and *RNR3*) and two β isoforms (encoded by *RNR2* and *RNR4*) (Elledge et al., 1993; Huang and Elledge, 1997).

RNR regulation, dNTP pools, and genomic integrity

The central role of RNR in controlling cellular dNTP pool highlights its importance in the maintenance of genome stability. Both the sizes and the relative ratios of cellular dNTP pools are critical for optimal DNA replication and repair. Abnormally elevated or imbalanced dNTP pools can lead to increased mutagenesis by enhancing mis-incorporation and inhibiting proofreading of DNA polymerases (Kunkel et al., 1982; Loeb and Kunkel,

1982; Reichard, 1988). Imbalance in dNTP pools can cause mutations, DNA breaks, and cell death (Ke et al., 2005; Song et al., 2005; Mathews, 2006; Kumar et al., 2010; Saldivar et al., 2012).

The regulation of RNRs is complicated and multifaceted, involving allosteric control, RNR subunit levels as well as subcellular localization. Allosteric regulation allows RNR to maintain an adequate and balanced supply of all four dNTPs and to adapt quickly to perturbation in cellular dNTP pools. The α subunit has two separate allosteric sites, S site and A site, which bind specific NTPs and dNTPs as effectors. The S site regulates specificity for each of the four substrates through binding of ATP, dATP, dTTP or dGTP. The A site controls overall enzyme activity through binding of ATP and dATP, with ATP binding activates, whereas dATP inhibits activity (Kashlan and Cooperman, 2003; Nordlund and Reichard, 2006).
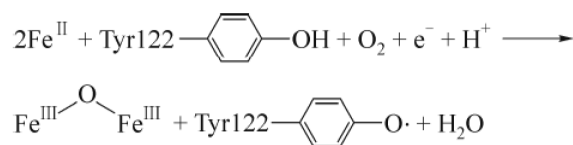
The RNR subunit protein levels are tightly regulated both by the cell cycle and the DNA damage checkpoint In proliferating cells, the dNTP pools increase 5- to 10-fold as cells transit from G_0/G_1 to S phase, which is mainly achieved by increased RNR levels at the G_1-to-S transition point (Mathews, 2006; Nordlund and Reichard, 2006). DNA replication occurs during the S phase and requires the continued synthesis of all four dNTPs. The RRM1 and RRM2 genes in mammals and plants are both maximally transcribed during S phase through the activity of the E2F family transcription factors (Björklund et al., 1990; Chabes et al., 2003b; Chabouté et al., 2000, 2002). In addition, RRM2 is also subjected to ubiquitin mediated pioteasomal degradation once cells completed DNA replication and/or repair, through two E3 ubiquitin ligase complexes, the Skp1/Cullin/F-box (SCF) and the anaphase-promoting complex (APC) (Chabes et al., 2003b; D'Angiolella et al., 2012). In budding yeast, transcription of the RNR genes peaks at the beginning of the S phase through regulation of the transcription factor pairs Mbp1/Swi6 and Swi4/Swi6 (Elledge et al., 1993; Sanvisens et al., 2013). In response to DNA damage, mammalian cells increase transcription of p53R2 (RRM2B) to facilitate DNA repair (Nakano et al., 2000; Tanaka et al., 2000) via activation of the ATM/ATR-CHK checkpoint pathway (Harper and Elledge, 2007). Similarly, DNA damage and replication blockage in budding yeast increase transcription of the *RNR* genes through activation of the Mec1-Rad53-Dun1 damage checkpoint kinase cascade. The activated checkpoint also removes two negative regulators of RNR in yeast cells, the Rnr1 inhibitor Sml1 and the Rnr2 nuclear transport facilitator Dif1 (Zhao et al., 2001; Lee et al., 2008; Wu and Huang, 2008), leading to a 6- to 8-fold increase of dNTP pool sizes (Chabes et al., 2003a; Chabes and Thelander, 2003).

Another mode of RNR regulation involves subcellular compartmentalization of the α and β subunits. In both the budding yeast and the fission yeast *Schizosaccharomyces pombe*, β is sequestered in the nucleus during most part of the cell cycle except for the S phase or in response to genotoxic stress, during which β relocalizes to the cytoplasm where a resides to allow holoenzyme formation (Liu et al., 2003; Yao et al., 2003). The nucleus-to-cytoplasm redistribution of β is mediated by the DNA damage checkpoint, which degrades Dif1 and Spd1, two small size proteins involved in nuclear import of β in *S. cerevisiae* and *S. pombe*, respectively. Regulation of RNR subunit subcellular localization has also been reported in mammalian and plant cells (Xue et al., 2003; Halimi et al., 2011). Interestingly, RRM1 and

RRM2 have recently been shown to accumulate at sites of DNA damage in mammalian cells, presumably to facilitate optimal localized dNTP supplies for repair (Niida et al., 2010; Hu et al., 2012).

The RNR metallocofactor: biosynthesis and maintenance

An important and yet much less understood layer of RNR regulation, given the fact that level of the Fe^{III}_2-Y• is directly correlated with nucleotide reduction activity of the holoenzyme (Ortigosa et al., 2006; Zhang et al., 2011), involves the assembly and maintenance of its metallocofactor. Early studies from the 1970s demonstrated that the Fe^{III}_2-Y• cluster in class Ia RNR can be self-assembled *in vitro* with apo-β_2, Fe^{II}, O_2, and reductant (Eq. (1)) (Atkin et al., 1973). The mechanism of self-assembly has since been elucidated by various rapid kinetics studies with wild-type and specifically engineered mutant β_2 proteins (Bollinger et al., 1991; Ormö et al., 1992; Salowe et al., 1993; Bollinger et al., 1995). A simplified model of RNR cofactor assembly is shown in Fig. 1 based on studies of the self-assembly process *in vitro*. The Fe^{III}_2-Y• cofactor assembly process involves delivery of Fe^{II} into β, reaction of the resulting Fe^{II}_2-β_2 with O_2 to form a diferric peroxide, and a series of intermediate steps that eventually require a one-electron reductant leading to oxidization of a conserved tyrosine, located near the di-iron center within β, to generate a tyrosyl radical Y• that can be measured by electron paramagnetic resonance (EPR) (Sjöberg and Reichard, 1977; Stubbe, 2003; Stubbe and Cotruvo, 2011). *In vitro* Fe^{II} serves both as the metal ligand and the reductant that provide the source of reducing equivalent for RNR cluster assembly. However, Fe^{II} is unlikely the election donor *in vivo* because it is highly reactive and has to be chaperoned inside the cell. The steps of Fe^{II} loading and election delivery are also poorly understood because of the inability to control these processes *in vitro*. The lack of control and general inefficiency of *in vitro* self-assembly argue for the importance of a cellular biosynthesis pathway for controlled cofactor formation.

$$2Fe^{II} + Tyr122\!-\!\!\bigcirc\!\!-\!OH + O_2 + e^- + H^+ \longrightarrow$$

$$Fe^{III}\!\overset{O}{\diagup}\!Fe^{III} + Tyr122\!-\!\!\bigcirc\!\!-\!O\cdot + H_2O$$

(1)

The Fe^{III}_2-Y• cofactor of eukaryotic RNR is inherently unstable, which raises the issue of cofactor repair, or namely, maintenance pathway. The half-life of the *E. coli* β_2 Y• is four days, whereas that in *S. cerevisiae* β_2 is several hours, the human β_2 25 min and the mouse β_2 10 min (Atkin et al., 1973; Thelander et al., 1983; Ortigosa et al., 2006; Hristova et al., 2008). The half-life of the RNR Y• is much shorter than the duration of the S phase of the cell cycle in mammalian cells. In addition, the RNR Y• is also susceptible to one electron reduction by endogenous and exogenous reductants such as hydroxyurea, resulting an inactive β_2 (met-β_2 with diferrictyrosine, i.e. Fe^{III}_2-Y). As such, there is a continual need for regeneration of the RNR Y• during S phase to meet the demand of dNTP production for genome replication during S phase (Barlow et al., 1983; Thelander et al., 1983). To

reactivate β_2 to produce deoxynucleotides, the Fe^{III}_2-Y• cofactor would need to be assembled either *de novo* (biosynthesis pathway in Fig. 1) from apo-β_2 or from half-way from met-β_2 (maintenance pathway in Fig. 1). It is conceivable that a cofactor maintenance/ repair process may play an important role for *in vivo* RNR regulation by modulating the levels of Y• /β_2 and consequently the nucleotide reduction activity.

The mechanism of RNR cofactor maintenance is under active investigation. The current model for the *E. coli* class Ia RNR maintenance pathway is that Fe^{III}_2-Y in met-β_2 can be reactivated by reduction of diferric (Fe^{III}_2) to diferious (Fe^{II}_2) form, which can subsequently form the active-β_2 through the biosynthetic pathway (Wu et al., 2007; Hristova et al., 2008). Early studies have demonstrated that the reduction of Fe^{III}_2 to Fe^{II}_2 could be achieved by a feriedoxin reductase Fie, NADPH, flavin (FAD or FMN), DTT, and a "Fraction B," the components of which remain to be defined (Coves et al., 1997; Fontecave et al., 1987, 1989). Recently, the [2Fe-2S]-ferredoxin protein YfaE has been shown to be involved in Fe^{III}_2-Y• cofactor maintenance in the *E. coli* class Ia RNR (Wu et al., 2007). It is unclear whether eukaryotic class Ia RNR employs similar cellular machinery for cofactor biosynthesis and maintenance. A problem with this notion is that the eukaryotic ferredoxin and ferredoxin reductase are primarily localized in the mitochondria (Lill et al., 2012).

RNR metallocofactor assembly: sources of iron and electron

The two key issues of *in vivo* RNR cluster assembly are the sources of iron and electron. Iron is an essential element for all eukaryotic organisms because iron cofactors, such as heme, iron-sulfur cluster, and di-iron clusters, are required for the activity of many cellular enzymes involved in a wide range of central cellular pathways including oxidative phosphorylation, ribosomal biogenesis, and DNA replication and repair (Rouault and Klausner, 1997; Kaplan et al., 2006; Ye and Rouault, 2010; White and Dillingham, 2012;). The facile ability of iron to gain and lose electrons allows it to participate in various oxidation-reduction reactions but on the other hand can lead to iron overload toxicity at higher levels (Rouault and Klausner, 1997). As such, cells have evolved sophisticated mechanisms for tightly regulated iron uptake, intracellular iron trafficking, and delivery of iron to specific proteins. The nature of bioavailable iron, namely the "labile iron pool," has been under debate since the 1960s (Pollycove and Maqsood, 1962). Recent studies have shown that two evolutionary conserved cytosolic monothiol glutaredoxins, Grx3/Grx4, are required to provide bioavailable iron to assembly of many iron-containing cofactors such as heme, Fe-S, and the di-iron cluster in RNR (Mühlenhoff et al., 2010; Zhang et al., 2011; Haunhorst et al., 2013). The monothiol glutaredoxins form a [2Fe-2S] bridged and GSH-liganded dimer, which mediates intracellular iron trafficking and utilization (Mühlenhoff et al., 2010). Cells depleted of Grx3/4 exhibit deficiencies in most iron-requiring reactions including the RNR nucleotide reduction activity, although it is unclear how Grx3/4 directly or indirectly participate in iron delivery into diverse iron-requiring proteins.

As for the source of the obligatory electron for RNR cofactor assembly, recent studies of the *S. cerevisiae* RNR suggest that the Fe-S cluster-containing protein Dre2 may act as the electron donor (Zhang et al., 2011). Dre2 and its binding partner, the diflavin NADPH oxidoreductase Tah18, (Netz et al., 2010) are conserved from yeast to human; their

mammalian counterparts are CIAPIN1 (or anamorsin) and NDOR1, respectively (Shibayama et al., 2004; Hao et al., 2005; Pujol-Carrion et al., 2006; Zhang et al., 2008). The two proteins constitute an electron transfer chain that transfers electrons from NADPH via its FAD and FMN to die Fe-S clusters of Dre2 (Netz et al., 2010). The cytosolic iron-sulfur cluster assembly (CIA) pathway, which is unique to eukaryotes and is for maturation of all cytosolic and nuclear Fe-S proteins, also require the Dre2-Tah18 complex (Netz et al., 2010), although the electron-requiring step requiring Dre2-Tah18 is yet to be identified. It would thus appear that the Dre2-Tah18 complex functions as the cytosolic equivalent of the mitochondrial ferredoxin-ferredoxin reductase pair to deliver electrons to multiple and divergent biosynthesis pathways of iron-containing cofactors. The findings that Grx3/4 and Dre2-Tah18 are required for RNR and the CIA pathway raise the intriguing perspective that biosynthesis of two apparently quite different iron cofactors, the diferric-Y• in RNR and the Fe-S cluster in nuclear and cytoplasmic proteins, share the same sources of iron and electrons (Fig. 2). It is conceivable that additional cytosolic and nuclear iron-requiring proteins also utilize these conserved protein complexes for cofactor maturation. In addition, physical and genetic interactions have been observed between Grx3 and Dre2 in yeast and mammalian cells consistent with a model that Dre2-Tah18 may also be involved in Grx3/4 Fe-S cluster maturation (Saito et al., 2011; Zhang et al., 2011).A mechanistic understanding of the contribution of Grx3/4 and Dre2-Tah18 to RNR cluster assembly would entail combination of genetic dissection and biochemical reconstitution studies.

Targeting the RNR metallocofactor in cancer treatment

The hallmark of cancer cells is uncontrolled proliferation (Hanahan and Weinberg, 2011). Cancer cells have heightened metabolic load and demand for energy and dNTP pools to replicate DNA (Cairns et al., 2011). RNR is a critical target in chemotherapy and increased RNR level and activity is associated with tumor growth, metastasis, and resistance to RNR-targeting drugs (Yen et al., 1994; Liu et al., 2007; Souglakos et al., 2008; Shao et al., 2013).

Although the human RRM1 (α) and RRM2 (β) are both required for the RNR holoenzyme activity, the two subunits exhibit different effect on cancer cells. In general, increased RRM2 expression is associated with enhanced invasive and metastatic potential and drug resistance of tumor cells, whereas RRM2 knockdown reverses drug resistance and suppresses proliferation (Fan et al., 1998; Souglakos et al., 2008; Zuckerman et al., 2011). In contrast, the role of RRM1 in cancer is more complex. Increased RRM1 expression is observed in many types of cancer. However, RRM1 overexpressionis often associated with reduced transformation and suppression of tumorigenicity and metastasis (Shao et al., 2013). The differences may reflect the potentially opposing effects of RNR-dependent dNTP biosynthesis on cancer cells at different stages of tumorigenesis. Improperly increased dNTP pools can reduce DNA repair fidelity and cause genomic instability. On the other hand, augmented dNTP pools may also enhance DNA repair capacity and survival of cancer cells, which are faced with increased replicational stress (Hanahan and Weinberg, 2011; Burrell et al., 2013).

RNR has been an important target in cancer treatment since the 1970s because of its central role in nucleotide metabolism (Brockman et al., 1970). Both small-molecule inhibitors and

antisense RNA therapy approaches have been successfully employed in RNR inhibition, which can enhance sensitivity to other chemotherapeutics and radiation therapy. Chemical inhibitors targeting the α or β subunit are the primary clinical strategy for RNR inhibition, α inhibitors are nucleoside analogs that inactivate enzyme activity through interaction with the substrate binding site or allosteric sites, whereas β inhibitors inactivate the metallocofactor (Cerqueira et al., 2007; Shao et al., 2013). In addition, the α–β interaction can be also targeted by using oligopeptides with similar sequences to the β C terminus, which interacts with a (Yang et al., 1990; Climent et al., 1991). Here, we will focus on several β metallocofactor targeting RNR inhibitors that are in clinical application or trials. These β inhibitors act by quenching the tyiosyl free radical (Hydroxyurea), depleting intracellular iron (Desferoxamine), or disrupting the di-iron center (Triapine).

Hydroxyurea is a simple small-molecule radical scavenger that has been used in cancer therapy for over five decades (Fishbein and Carbone, 1963). It inactivates RNR by directly reducing the tyrosyl radical via one-electron transfer from hydroxyurea to Y• and causing structural perturbation of the di-iron center of β (Lassmann et al., 1992; Offenbacher et al., 2009). Hydroxyurea treatment depletes cellular dNTP pools leading to stalled replication forks and activation of the DNA damage checkpoint (Bianchi et al., 1986; Allen et al., 1994; Davies et al., 2009). Hydroxyurea has been widely used for treatment of acute and chronic myeloid leukemias (AML and CML), head and neck cancers, and brain tumors (Donehower, 1992; Kennedy, 1992; Madaan et al., 2012). Furthermore, the synergism of hydroxyurea with nucleoside analogs didanosine (DDI) and 3′-azido-3′-deoxythymidine (AZT) has shown effectiveness for treatment of HIV-1 infectionin clinical trials, likely due to their effect on alteration of the cellular dNTP pools (Johns and Gao, 1998; Mayhew et al., 2005). Although β is a major target of hydroxyurea, other metalloenzymes can also be inhibited by hydroxyurea (Scozzafava and Supuran, 2003; Campestre et al., 2006). In addition to its promiscuity, the effectiveness of hydroxyurea is limited by its low affinity for RNR short half-life and the development of resistance due to β overexpression (Yen et al., 1994; Gwilt and Tracewell, 1998; Ren et al., 2002).

Desferoxamine is a bacterial siderophore that is used clinically as an iron chelator in treatment of iron over-load disorders and acute iron poisoning. It acts by binding free iron in the bloodstream and facilitating iron removal (Miyajima et al., 1997). Desferoxamine has antiproliferative effect both *in vitro* and *in vivo*, which is mediated by its depleting effect on intracellular iron pools (Dayani et al., 2004). Since iron is an essential component of the RNR Fe^{III}_2-Y• cofactor, depletion of intracellular iron pool would impair RNR cofactor biosynthesis. In mammalian cells, iron deprivation results in decreased RNR activity, leading to cell growth arrest (Nyholm et al., 1993). It has been shown that desferoxamine inhibits RNR activity through chelating intracellular iron pools and preventing formation of the RNR Fe^{III}_2-Y• cofactor, which can be reversed by addition of iron (Chaston et al., 2003). Since iron is a cofactor of many enzymes in addition to RNR, the effect of desferoxamine treatment is pleiotropic. The clinical efficacy of desferoxamine is also limited by its poor membrane permeability and a very short half-life in the bloodstream (Chaston and Richardson, 2003; Lovejoy and Richardson, 2003). This limitation has encouraged the

development of more effective iron chelators with novel properties, one of which is Triapine.

Triapine (3-AP) is a potent β inhibitor that is currently in phase II clinical trials for treating CML and various solid tumors (Finch et al., 1999; Kunos et al., 2012; Zeidner et al., 2013). It belongs to the heterocyclic carboxaldehyde thiosemicabazone family of iron chelators, which exhibit wide-spectmm antitumor activity and cytotoxicity (Buss et al., 2003; Yu et al., 2006). Studies to date suggest that the cytotoxicity of 3-AP involved multiple mechanisms. 3-AP chelates iron by forming complexes with both Fe^{II} and Fe^{III} (Enyedy et al., 2011). The Fe^{II}-(3-AP) can react with O_2 to produce reactive oxygen species (ROS) (Richardson et al., 2009). Based on these properties of 3-AP, several models have been proposed for the mechanism of β inhibition by 3-AP. One model suggests that 3-AP chelate Fe^{III} directly from the β Fe^{III}_2-Y• cofactor leading to RNR inactivation. Another model proposes that 3-AP chelates iron from the intracellular iron pool (s), thus interfering with the β cofactor biosynthesis. A third model posits that Fe^{III}-(3-AP) resulted from Fe^{III} chelation is reduced to Fe^{II}-(3-AP) by endogenous reductants, which subsequently leads to ROS in the presence of O_2 causing β inactivation an DNA degradation. Recent biochemical studies have revealed that the loss of the Y• signal, measured by EPR, occurs independently of O_2 and ROS and always precedes the loss of iron when $β_2$ is incubated with 3-AP (Aye et al., 2012b). These results indicate that the observed rapid inhibition of β activity by 3-AP results from reduction of Y• by Fe^{II}-(3-AP), although the source of the Fe^{II}-(3-AP) remains to be determined (Aye et al., 2012b). 3-AP can inhibit both RRM2 and p53R2 of the mammalian RNR, whereas hydroxyurea is ineffective at inhibiting p53R2 (Shao et al., 2004; Yu et al., 2006). The Fe^{II}-(3-AP) complex has been reported to be more effective at inhibiting RNR than free 3-AP (Shao et al., 2006), consistent with the notion of Y• reduction by Fe^{II}-(3-AP) as the main mechanism of rapid RNR inhibition by 3-AP.

Conclusions

RNR is uniquely positioned as the rate-limiting step in *de novo* dNTP biosynthesis required for DNA replication and repair and hence an important therapeutic target. RNR level and activity is tightly regulated both during the cell cycle progression in proliferating cells and in response to genotoxic stress in all cell types. The molecular details of RNR regulation, which involves transcription, protein degradation and subcellular localization, have emerged from both biochemical and genetic studies in model organisms and cell cultures. A much less understood mode of RNR regulation is at the level of its metallocofactor assembly, a process that requires sources of bioavailable iron and reducing equivalents. Recent studies in *S. cerevisiae* have identified the monothiol glutaiedxoins Grx3/4 and the Fe-S cluster containing protein Dre2 as potential sources of iron and electrons, respectively, in RNR cofactor assembly. Delineation of the steps involved in RNR cofactor biosynthesis demands future biochemical and genetics studies. The inherent instability of the Y• of eukaryotic RNR suggests the existence of a maintenance pathway that sustains RNR activity to provide adequate dNTP pools for completion of DNA replication during each cell cycle. Several classes of RNR inhibitors targeting different components of the holoenzyme have been in clinical use and trials. A detailed mechanistic understanding of RNR cofactor assembly may

provide critical insights into future development and investigation of RNR-targeting therapeutics with higher specificity and selectivity.

Acknowledgments

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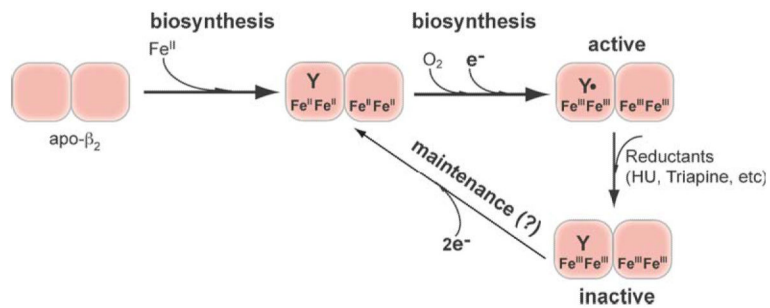
Figure 1.
The proposed pathways for biosynthesis and maintenance of the Fe^{III}_2-Y• cofactor of class Ia RNR. The biosynthetic pathway requires delivery of two Fe^{II} and a reducing equivalent (electron) per β subunit to carry out the four-electron reduction of O_2 to H_2O (Eq. (1)). The other three electrons come from the two Fe^{II} and Tyr residue to form the Fe^{III}_2-Y•. The maintenance pathway may use the same source of reducing equivalents to convert the inactive Fe^{III}_2-Y cluster to Fe^{II}_2-Y, which subsequently forms Fe^{III}_2-Y• in the presence of O_2 via the biosynthetic pathway.
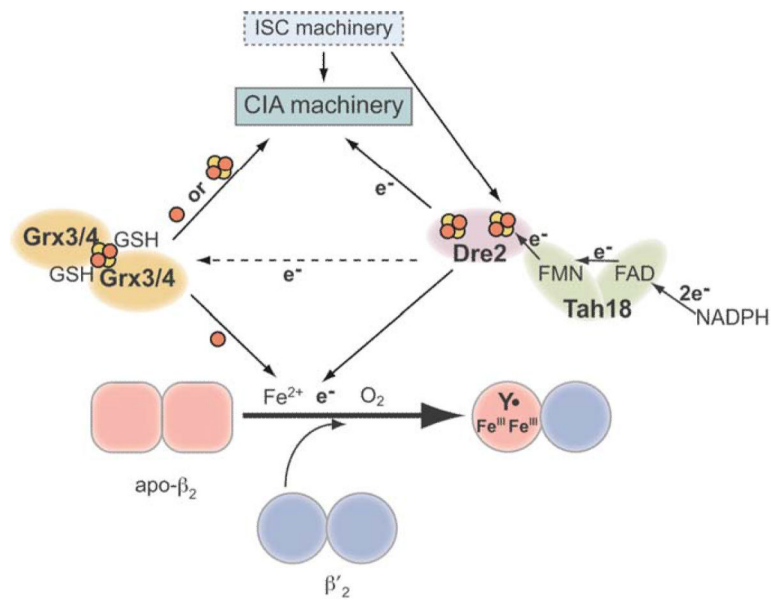
Figure 2.
A model depicting the role of the Dre2-Tah18 complex as the electron donor for cluster assembly in both RNR and in cytosolic and nuclear Fe-S cluster containing proteins. Grx3/4 is the source of iron for all cellular iron-requiring pathways including Fe-S cluster assembly in mitochondria (ISC) and cytosol (CIA), and Fe^{III}_2-Y• assembly in RNR (Mühlenhoff et al., 2010). Electrons from NADPH are transferred via FAD and FMN, the two flavin cofactors in Tah18, to the Fe-S cluster(s) in Dre2, which subsequently deliver the electrons to proteins in the CIA pathway and β in RNR. Dre2-Tah18 may also provide reducing equivalents to facilitate iron release from the [2Fe2S]-GSH_2 cluster in the Grx3/Grx4 dimer (Zhang et al., 2011).