Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase

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Ribonucleotide reductase (RNR), the rate-limiting enzyme in DNA synthesis, catalyzes reduction of the different ribonucleotides to their corresponding deoxyribonucleotides. The crucial role of RNR in DNA synthesis has made it an important target for the development of antiviral and anticancer drugs. Taking account of the recent developments in this field of research, this review focuses on the role of thioredoxin and glutaredoxin systems in the redox reactions of the RNR catalysis.

INTRODUCTION

Ribonucleotide reductase (RNR) catalyzes the rate limiting step of the DNA synthesis where the reduction of ribonucleotides (NTPs) results in the formation of corresponding deoxyribonucleotides (dNTPs). The RNR catalysis involves protein free radicals, redox-active thiois and proteins of the thioredoxin (Trx) superfamily. In the RNR complex, the R1 subunit contains the active site, allosteric sites and redox active thiols/disulfides required for the RNR catalysis; while the R2 subunit provides a dinuclear metal cluster and a tyrosyl free radical essential for the catalytic cycle. The RNR activity can be regulated by expression of different subunits, subcellular localization, post-translational modifications and allosteric regulation involving both activity and substrate specificity.

The DNA replication is coordinated with the cell growth by different regulatory mechanisms. Development of malignancy and cancer are found to be associated with an increased expression and activity of RNR. In cells, an imbalance in the levels of dNTPs will cause mutagenesis and carcinogenesis. On the other hand, blockage of RNR activity can inhibit DNA synthesis and repair which results in apoptosis. In recent years, several RNR inhibitors have entered clinical trials. Recent developments in the field will provide a new basis for the discovery of more
effective RNR inhibitors for cancer therapy. Taking account of the recent progress in this field of research, this review focuses on the role of Trx and glutaredoxin (Grx) systems in the redox regulations of the RNR catalysis.

**RNR: CLASSIFICATION AND CATALYSIS**

Based on the pathways of radical initiation and requirements of metal cofactors, the RNRs have been divided into three classes\[2-5,9\]. The active site of all three classes of RNR has a very similar structure (Figure 1)\[2-5,9\]. A conserved cysteine residue plays the vital role for the generation of a thyl radical in all the classes of RNR (Figures 1 and 2). The reaction mechanisms of different classes of RNR are similar due to the structural similarities of the catalytic domains. The RNR catalysis starts with the generation of a thyl radical close to the bound substrate\[2-5\]. Then the abstraction of hydrogen from the C3' of the ribose ring and generation of a substrate radical occurs. During the RNR catalysis, a cysteinyl radical, required for the abstraction of hydrogen at the C3' ribose substrate, is derived from a tyrosyl radical for class I or cobalamin cofactor for class II or a glycy radical for class III. For class I and II enzymes, electrons required for the reduction of the ribonucleotides are provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) through Trx or Grx systems. However, for class III enzymes, the electrons are supplied by formate.

The class I RNR occurs in eukaryotes, eubacteria, bacteriophages and viruses. The complex, in its simple form, is a tetramer with the dimer of larger subunit (R1) and the dimer of R2 subunit (Figures 1 and 2)\[2-5\]. Oxygen is required for the generation of a tyrosyl radical (Tyr 122) in the R2 subunit. As described above, during catalysis, the radical is continuously transferred to a cysteine (Cys 439) residue of the R1 subunit and generates a thyl radical to activate the substrate. The R1 subunit carries the catalytic site, allosteric effector binding sites and redox-active thiol groups required for the catalysis. The R1-p53R2 complex has been found to play a significant role in DNA repair and the R1–R2 complex is suggested to be associated with DNA replication\[11\]. Moreover, the R1-p53R2 complex is suggested to be required for basal DNA repair and the R1–R2 complex is suggested to be associated with DNA replication\[11\]. The expression of the p53R2 subunit is induced by DNA damage which is mediated by the tumor suppressor protein p53. Both R2 and p53R2 subunits use a diferric iron center generating a tyrosyl free radical required for the RNR catalysis. The R1-p53R2 complex is suggested to be required for basal DNA repair and the R1–R2 complex is suggested to be associated with DNA replication\[11\].

For class II RNR (archaebacteria, eubacteria), a cofactor (5'-deoxyadenosylcobalamin) replaces the presence of a separate subunit for storage of radicals\[2-5\]. The cleavage of 5'-deoxyadenosylcobalamin generates a deoxyadenosyl radical which abstracts hydrogen from the active site cysteine residue. Trx system can reduce the C-terminal pair of redox-active cysteines which, in turn, can reduce the active site to continue the RNR catalysis. For anaerobic class III RNR (archaebacteria, eubacteria, bacteriophages), a glycy radical is generated by the action of activase, S-adenosylmethionine and a reducing factor (5'-deoxyadenosylcobalamin) replaces the presen of a separate subunit for storage of radicals. WJBC www.wjgnet.com 69 February 26, 2014 Volume 5 Issue 1
system containing flavodoxin, NADPH and NADPH-flavodoxin reductase. Then the glycol radical generates a thyl radical required for the catalysis.

**TRX AND GRX SYSTEM**

Trx is a class of 12 kDa ubiquitous redox proteins found primarily in the cytosol[13]. Trxs possess a catalytically active dithiol function in a Cys-Gly-Pro-Cys motif and are present in all organisms. The complete mammalian Trx system comprising Trx, the selenoenzyme thioredoxin reductase (TrxR) and NADPH also plays a crucial role in redox signaling and thiol homeostasis of cells. Cytosolic Trx1 and mitochondrial Trx2 regulate several metabolic pathways, oxidative/nitrosative stress defence, apoptosis and DNA synthesis[4,13-15]. On the other hand, Grxs are small redox enzymes of approximately 10 kDa and they participate in thiol-disulfide exchange reactions in the presence of glutathione (GSH), glutathione reductase (GR) and NADPH. In the Grx system, Grx is reduced via GSH[16]. The glutathione disulfide formed is then reduced by GR and NADPH. Grxs are involved in redox signaling and maintenance of cellular redox environment. Moreover, GSH synthesis was found to be essential for mouse development[19], whereas the deletion of Trx gene was reported to be lethal for mouse embryos[20]. Loss of TrxR1 showed no effect on the normal replicative potential[21]. However, the survival of TrxR1-deficient tumor cells was found to be very much dependent on GSH[22]. In a recent study, it has also been shown that the GSH/Grx system can reduce Trx1 in TrxR1-deficient HeLa cells[23].

**ROLE OF TRX AND GRX AS EXTERNAL ELECTRON DONORS FOR RNR**

Trxs and Grxs belong to related families of low molecular weight redox enzymes catalyzing thiol-disulfide exchange reactions with catalytically active cysteine thiols in a CXXX active site[13,16]. For class I and II RNR enzymes, the electrons are supplied by NADPH through Trx or Grx systems[2-5]. Reduction of ribonucleotide in the RNR catalysis involves the formation of a disulfide in the active site of R1 subunit. Structural studies with E. coli RNR show that the active site cleft of the R1 subunit is not very wide to permit the direct reduction by the external redox system[9,124]. However, the reduction of active site disulfide is performed by a pair of shuttle cysteine residues in the C-terminal mobile tail of R1 subunit (Figure 3)[4,25,26]. The C-terminal shuttle dihithiols of E. coli R1 subunit has the CXXXXC sequence; whereas yeast and mammalian R1 has a CXXXC sequence. In vitro
mutagenesis and kinetic studies support a critical role for the C-terminal cysteine pair of R1 in regeneration of the active site\cite{27,28}. The disulfide exchange reaction results in the formation of a disulfide in the C-terminal tail of R1. Then, the external redoxin systems reduce the disulfide bond to continue the next cycle of RNR catalysis.

Trx and Grx systems were found to act as dithiol electron donors of \textit{E. coli} RNR\cite{32-34}. Recently, Gustafsson et al\cite{31} characterized the Trx1 system as the physiologically relevant electron donor for RNR in \textit{Bacillus anthracis}. In \textit{E. coli}, the class 1a enzyme requires the dithiol form of at least one of Grx1, Trx1 or Trx2 to be viable\cite{32-34}. The Grx1 system showed 10-fold lower \(k_{\text{cat}}\) value compared to that of Trx1 system, while both of the redoxins had similar \(v_{\text{max}}\) (Table 1). This makes Grx1 the most efficient electron donor for the \textit{E. coli} enzyme. However, there is a mechanistic difference between the \textit{E. coli} and the mammalian RNR catalysis involving Trx1 and Grx1 systems as electron donors\cite{30}. Trx1 and Grx1 system showed similar catalytic efficiencies (\(k_{\text{cat}}/k_{\text{m}}\)) with recombinant mouse RNR complex (Figure 4 and Table 1). In the presence of 4 mmol/L GSH, the Grx1 system showed a higher affinity compared to Trx1 and displayed a higher apparent \(k_{\text{cat}}\). The RNR activity with the Grx system was found to be very much dependent on the concentrations of GSH. Here, it is noteworthy to mention that mammalian cells have significantly high concentrations of GSH (5-20 mmol/L)\cite{34}. Moreover, the ability of the monothiol mutant of Grx2 to maintain RNR catalysis demonstrates a glutathionylation mechanism for Grx catalysis in contrast to the dithiol mechanism for the Trx system\cite{30}. However, the \textit{E. coli} RNR complex showed no activity with the monothiol mutant of bacterial Grx1 suggesting the involvement of a dithiol-disulfide mechanism for the catalysis\cite{30}.

The advantage of a glutathionylation mechanism may be with the very low levels of R1 involved in the repair and production of dNTPs for mitochondrial DNA synthesis. Trx is present at low levels in many resting postmitotic cells. The sigmoidal curve of Trx activity showed that reduced Trx could not be efficient with a low concentration of R1 in postmitotic cells\cite{30}. The high concentration of GSH\cite{30} would ensure that there is glutathionylated R1 and then monothiol/dithiol Grx should be able to catalyze reduction of the C-terminal disulfide. Several studies reported that the rapidly proliferating cells have increased GSH concentration, while a decrease in GSH concentration limits cell proliferation. GSH acts as a key regulator of cell proliferation and thus the colocalization of GSH with nuclear DNA was observed in proliferating cells\cite{37}. In mammary carcinoma cells, the depletion of glutathione was found to inhibit DNA synthesis\cite{38}. A similar study with 3T3 fibroblast cells showed a significant correlation between progression of cell cycle and the distribution of nuclear GSH\cite{39}. Moreover, accumulation of DNA damage was found in liver, kidney and lung of mice deficient in \(\gamma\)-glutamyl transpeptidase, the enzyme responsible for initiating the catabolism of GSH\cite{38}. In another study, down-regulation of TrxR showed no effects on the dNTP pools in malignant mouse cells\cite{38}. This suggests the role of the GSH/Grx system as an alternative pathway used by the RNR in tumor cells. Moreover, a study in mouse hepatocytes, suggested the importance of a TrxR-independent pathway for the supply of electrons

\begin{figure}
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{The mechanistic model for the role of thioredoxins and glutaredoxins for the ribonucleotide reductase catalysis. After the completion of one turn-over cycle of ribonucleotide reductase (RNR) catalysis, a disulfide bond is formed between the conserved cysteine pair at the active site (shown in the circle). Shuttle dithiol function present at the C-terminal CXXC motif of the neighboring subunit reduces the disulfide bond through disulfide-exchange. Then, the resulting disulfide bond at the C-terminal tail is reduced by the thioredoxin/glutaredoxin (Trx/Grx) systems resulting in an active R1 to continue the next cycle of RNR catalysis. The Grx system can also reduce the C-terminal thiols by the glutathionylation mechanism\cite{27,28}. For simplicity, only the reduction of active site of one subunit by the C-terminal shuttle dithiols of the neighboring subunit is shown in the diagram. The figure is adapted and modified from Holmgren et al\cite{4}.}
\end{figure}
to RNR. These studies clearly show the crucial role of the GSH/Grx system in DNA repair via RNR catalysis.

ROLE OF TRX FOR CLASS III RNR

The class III RNR (present in strict and facultative anaerobes) forms an inactive αβε complex in resting state. The cysteine residues present in the C-terminus of protein α were found to be responsible for the formation of glycy radical and to participate in radical transfer reactions during enzyme activation. Under the reducing condition, the small β subunit can activate several α proteins. The Trx system was found to activate the enzyme with the same efficiency as dithiothreitol (DTT). The data suggests that the Trx system keeps the conserved cysteines of the C-terminus of the α-polypeptide in a reduced form which is required for radical generation. Therefore, Trx acts only for the activation of the class III RNR. Later, a structural study of the homologous enzyme from bacteriophage T4 revealed the presence of zinc bound to four conserved cysteine residues. It was also shown that the Trx system or DTT is dispensable for the formation of the glycy radical with the fully Zn-loaded RNR. The radical transfer from glycine to the active-site cysteine to the substrate is controlled by a crucial hydrogen-bond network. Thus, the suggested role of the Trx system (or DTT) was to facilitate the recognition of the network and allow efficient radical transfer.

CONCLUSION

For several years, the RNR inhibitors have been used to treat cancers and viral infections. Most of the RNR inhibitors are either radical scavengers (hydroxyurea) or metal chelators (tripamine) which specifically inactivate the R2 subunit. On the other hand, several nucleoside analogs and sulfhydryl group inhibitors (such as cisplatin, caracemide, chlorambucil, etc.) are used as R1-specific inhibitors. Gene expression silencers and R1-R2 polymerization inhibitors (oligopeptides) have also been used to block RNR activity. In recent years, many new strategies have emerged in the designing of subunit-specific and more effective RNR inhibitors. Redox regulation of RNR catalysis plays a vital role as the RNR catalysis involves different redox active thiol functions, thiol radicals and thiyl proteins of the Trx superfamily.

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Table 1 Kinetic parameters of thioredoxin 1, glutaredoxins (1, 2, 2C40S) for reduction of mouse ribonucleotide reductase complex

| Electron donor system | Vmax (μmol/L per second per microgram of R1) | kcat (μmol/L) |
|-----------------------|---------------------------------------------|---------------|
| Trx1                  | 2.2                                         | 1.90          |
| Grx1                  | 2.3                                         | 0.18          |
| Grx2                  | 1.5                                         | 0.30          |
| Grx2C40S              | 1.5                                         | 0.36          |

Data adapted from Zahedi Avval et al. [24]. Trx: Thioredoxin; Grx: Glutaredoxins.
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