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Studies on Ribonucleotide Reductase, a Target for Anticancer Therapy, with Focus on Electron Donor Aspects -Thioredoxin and Glutaredoxin Systems

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Cover pictures show cytoplasmic localization of R1 (*red*) and R2 (*green*) subunits.

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To my family

ABSTRACT

Faithful replication of DNA and its repair are fundamental processes in all living cells which utilize deoxyribonucleotides as DNA building blocks. Ribonucleotide reductase (RNR) is the essential enzyme for *de novo* synthesis of these precursors from ribonucleoside diphosphates. Each catalytic cycle of RNR, requires disulfide bond reduction which as shown in *Escherichia coli*, is catalyzed by thioredoxin (Trx) or glutaredoxin (Grx) systems. These are general protein disulfide reductases, and the presence of one system is essential. The Trx system comprises Trx, Trx reductase (TrxR), and NADPH; and the Grx system is composed of Grx, glutathione (GSH), glutathione reductase, and NADPH. This thesis studies mammalian RNR; in S-phase cells RNR comprises a weak complex of a catalytic R1 protein, containing redox active cysteine residues, and a radical generator subunit termed R2.

By analyzing recombinant mouse RNR with respect to electron donors, we found that Trx1 and Grx1 had similar catalytic efficiency (k_{cat}/K_m). With 4 mM GSH, Grx1 showed a higher affinity (apparent K_m-value 0.18 μM) compared to Trx1 which displayed a higher apparent k_{cat} suggesting its major role in S-phase DNA replication. Surprisingly, Grx activity was strongly dependent on GSH concentrations (apparent K_m-value 3 mM), and a Grx2 Cys40Ser mutant was active despite only one cysteine residue in the active site. These results demonstrate a GSH-mixed disulfide mechanism for Grx catalysis, in contrast to the dithiol mechanism for Trx. We propose that this may be an advantage with the low levels of RNR for DNA repair or in tumor cells with high RNR and no or low Trx expression.

Different isoforms of mouse Grx2 were identified, which were further characterized with respect to subcellular localization, expression pattern, and enzymatic activity. Amoung three different isoforms (Grx2a, Grx2c, and Grx2d), mitochondrial Grx2a was expressed in all tissues, except testis. On the other hand, Grx2c and Grx2d were cytosolic and expressed in testis. Mouse Grx2c had general Grx-activity and could reduce RNR, but Grx2d lacked enzymatic activity. These data provide evidence for additional functions of Grx2 in the cytosol, in cell proliferation, and in cellular differentiation.

Motexafin gadolinium (MGd) is a new anticancer agent with promising results in clinical trials, which selectively targets tumor cells and works as a radiation enhancer. It mediates redox reactions generating reactive oxygen species (ROS) with oxidation of intracellular reducing molecules. MGd was an NADPH-oxidizing substrate for TrxR. The reaction involved redox cycling of MGd by oxygen producing superoxide. MGd acted as a non-competitive inhibitor for TrxR. In contrast, direct reaction between MGd and reduced Trx was negligible. MGd inhibited recombinant RNR activity with either the Trx system or dithiothreitol as electron donors. Overall, our results show that MGd induces generation of ROS by TrxR and is a powerful inhibitor of RNR.

Further studies on the mechanism of inhibition of RNR by MGd, revealed at least two different mechanisms for its effect: interruption in subunit oligomerization, and direct inhibition of the catalytic subunit (R1). Co-localization of MGd and RNR in the cytoplasm was shown particularly in the S-phase. These data elucidate another important effect of MGd on the cancer cells with overproduction of RNR, and highlights its efficacy as an anticancer agent.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the thesis by their Roman numerals.

I. Hashemy, S.I., Ungerstedt, J. S., **Zahedi Avval, F.**, and Holmgren, A.
Motexafin gadolinium, a tumor-selective drug targeting thioredoxin reductase and ribonucleotide reductase. *J. Biol. Chem.*, 281: 10691-7 (2006).

II. Hudemann, C., Lönn, M.E., Godoy, J.R., **Zahedi Avval, F.**, Capani, F., Holmgren, A., and Lillig, C.H.
Identification, expression pattern, and characterization of mouse glutaredoxin 2 isoforms. *Antioxid. Redox Signal*., 11: 1-14 (2009).

III. **Zahedi Avval, F.**, Berndt, C., Pramanik, A., and Holmgren, A.
Mechanism of inhibition of ribonucleotide reductase with motexafin gadolinium (MGd). *Biochem. Biophys. Res. Commun.,* 379: 775-9 (2009).

IV. **Zahedi Avval, F.** and Holmgren, A.
Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for mammalian S-phase ribonucleotide reductase. *J. Biol. Chem., In Press*.

CONTENTS

LIST OF ABBREVIATIONS

Cys (C)	*Cysteine*
DNCB	*1-Chloro-2, 4-dinitrobenzene*
dNDP	*Deoxyribonucleoside diphosphate*
dNTP	*Deoxyribonucleoside triphosphate*
DTNB	*5, 5´-Dithiobis (2-nitrobenzoic acid)*
DTT	*Dithiothreitol*
FCS	*Fluorescence correlation spectroscopy*
Gly	*Glycine*
GR	*Glutathione reductase*
Grx	*Glutaredoxin*
GSH	*Glutathione*
GSSG	*Glutathione disulfide*
k_{cat}	*Turnover number*
K_m	*Substrate concentration when velocity is half of* V_{max}
MGd	*Motexafin gadolinium*
MRI	*Magnetic resonance imaging*
NADPH	*Nicotinamide adenine dinucleotide phosphate*
NDP	*Ribonucleoside diphosphate*
NTP	*Ribonucleoside triphosphate*
Phe	*Phenylalanine*
Pro	*Proline*
p53R2	*R2 homologue*
R1	*Large subunit of RNR*
R2	*Small subunit of RNR*
RNR	*Ribonucleotide reductase*
ROS	*Reactive oxygen species*
Sec	*Selenocysteine*
Ser	*Serine*
Trx	*Thioredoxin*
TrxR	*Thioredoxin reductase*
Tyr	*Tyrosine*
V_{max}	*Maximum velocity*

1 INTRODUCTION

1.1 BACKGROUND

Deoxyribonucleic acid (DNA), the giant macromolecule encoding the genetic information of all living cells, cannot be replicated without a balanced supply of its building blocks, deoxyribonucleoside triphosphates (dNTPs). The nuclear DNA in the cell is organized in chromosomes. Eukaryotic cells also have a separate small DNA genome in their mithochondria. While the nuclear DNA replication occurs only in proliferating cells during the S-phase of the cell cycle, the mitochondrial DNA replication is active independently of the cell cycle [1].

Nucleotides are composed of a nitrogen-containing carbon ring (the base) coupled to a phosphorylated five-carbon sugar (ribose). The bases of DNA are heterocyclic aromatic rings, with a variety of substituents (Fig. 1a); adenine (A) and guanine (G) are purines with bicyclic structure, whereas thymine (T) and cytosine (C) are monocyclic pyrimidines. In DNA the sugar is deoxyribose, hence the 2′–OH group in ribose (in RNA structure) is replaced by a hydrogen atom (Fig. 1b).

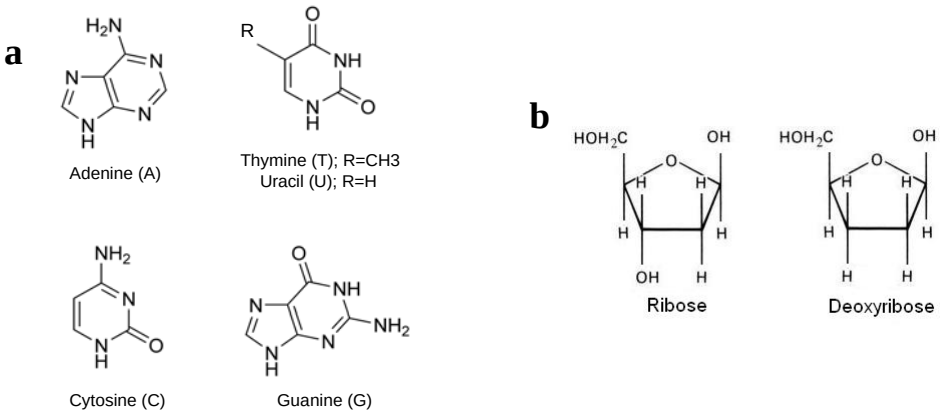

Figure 1. (a) Nucleic acid bases; (b) sugar moiety of RNA and DNA.

Ribonucleoside monophosphates are initially synthesized from low molecular weight precursors like amino acids, NH_4^+, CO_2, and ribose [2], and further phosphorylated to their diphosphate counterpart by nucleoside monophosphate

kinases [3]. However, there is no corresponding synthetic pathway for dNTPs; moreover, diffusion over the plasma membrane is prevented by the negative charge of the phosphate groups and there are no carrier proteins for nucleotides in the cell membrane. The different pathways by which the dNTPs can be synthesized in the cell are, therefore, the *de novo* and the salvage pathways. All *de novo* synthesis is catalyzed by a ubiquitous enzyme, ribonucleotide reductase (RNR) which reduces ribonucleotides to the corresponding deoxyribonucleotides [4]. In the salvage pathway the deoxyribonucleosides are imported through nucleoside carrier proteins from extracellular space and then phosphorylated to the corresponding deoxyribonucleoside monophosphates. This reaction is catalyzed by deoxyribonucleoside kinases [5, 6].

The dNTP can diffuse freely through the nuclear pores thus creating an equilibrium of the dNTPs between the cytosol and the nuclei. The four pools have different sizes with the dTTP pool normally being the largest and dGTP the smallest [7]. Mithochondria have been shown to have dNTP pools that are separated from the pools in the cytosol. However, they are in exchange via mitochondrial transporters [8], and the mitochondrial pools reflect the size of the cytosolic ones [9].

1.2 RIBONUCLEOTIDE REDUCTASE

The conversion of ribonucleotides into the correspondent deoxyribonucleotides plays a central role in nucleic acid metabolism. Ribonucleotide reductase (RNR) discovered by Peter Reichard in the 1950s, catalyzes the biosynthesis of the four deoxyribonucleotides. The enzyme contains two subunits: a reductase and a radical generator, named R1 and R2, respectively; both components are required for enzyme activity (Fig. 2) [4, 10, 11].

The actual reduction of the substrate is performed by one pair of cysteines at the active site of R1 which are oxidized during the reaction. The involvement of cysteine residues in the reduction of ribonucleotides was recognized with showing that one disulfide bridge is formed for each ribonucleotide reduced, and that two ribonucleotides can be reduced by the fully reduced protein in the absence of external reductants, indicating the involvement of two disulfide bridges in the active monomer of R1 class Ia RNR from *E. coli* [12].

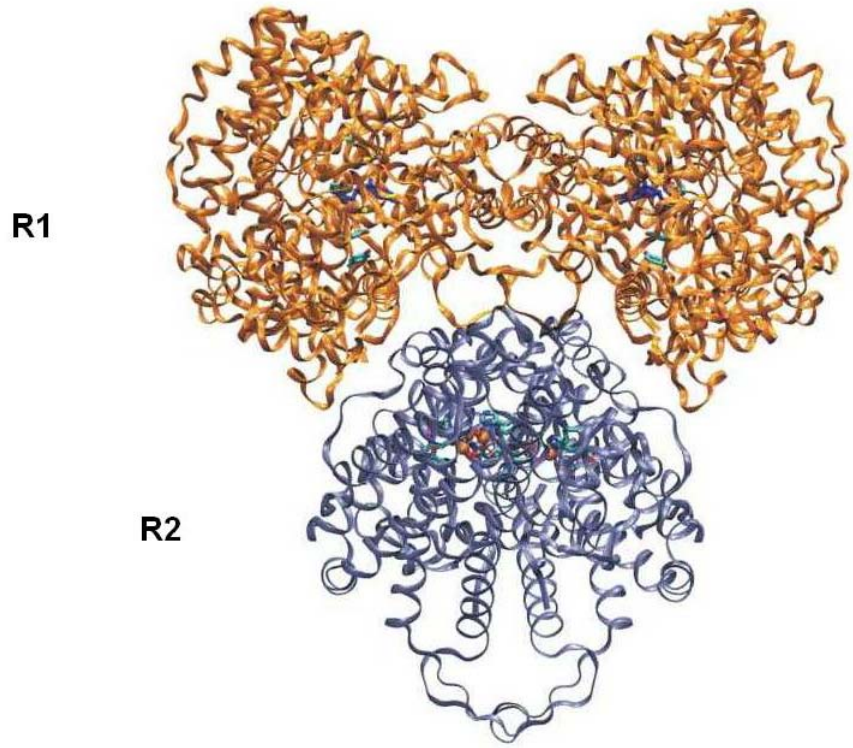

Figure 2. Structural model of the R1-R2 holoenzyme of *E.coli* RNR.

RNR is the only enzyme described in detail cysteines can reduce a carbohydrate substrate. Usually cysteine residues reduce other thiol compounds by simple thiol-disulfide exchange reactions. The active site disulfide has to be reduced before the enzyme can go through another cycle of substrate reduction [11, 13]. The two well-known pathways which assist RNR in *Escherichia coli* are thioredoxin and glutaredoxin systems. In the first one, the oxidized thioredoxin is reduced by thioredoxin reductase, and in the second, glutaredoxin is reduced by glutathione, which is in turn reduced by glutathione reductase; in both pathways, NADPH is the ultimate reductant [14]. While dicysteinyl proteins like thioredoxin and glutaredoxin serve as natural hydrogen donors of RNR; dithiols of low molecular weight such as dithiothreitol (DTT) can function *in vitro*.

As a key enzyme, the activity of RNR is highly transcriptionally regulated and is cell cycle dependent [15, 16]. Moreover, the overall enzymatic activity is regulated by ATP as a general activator, and dATP as a feedback inhibitor acting at high concentrations [17]. Although, several models have been proposed, it is not known how the two almost identical nucleotides (ATP or dATP) can have opposite effects on overall enzyme activity [18-20]. To provide a balanced supply of the different

deoxyribonucleotides, RNR has a unique additional allosteric regulation in such a way that binding of triphosphate nucleotides regulate its substrate specificity. The specificity site, also located on R1, interacts with ATP, dATP, dTTP or dGTP and determines the substrate to be reduced [21, 22].

1.2.1 Classification

RNRs in living cells can be grouped into three different classes (I, II and III), mainly based on oxygen dependence, different cofactors used for the catalytic activity, and mechanism for the radical generation (Table 1) [23, 24]. Based on differences in allosteric regulation and polypeptide sequence, class I RNRs are further subdivided into class Ia and Ib. Class Ia enzymes have an overall activity site while class Ib lacks the N-terminal region where this site is located [25].

All RNRs share a common basic catalytic mechanism, involving the initiation of the reaction by removal of the 3'-hydrogen of the ribose by a transient cysteinyl radical [26]. The radical is formed at a di-iron site in the R2 subunit; stored as a tyrosyl radical and delivered to the R1 as a transient radical when needed for catalysis [10].

As mentioned above, one of the main criteria for classification is the way of radical generation [17]. In class Ia a stable tyrosyl radical through the action of a binuclear iron center is produced, enzymes in class II use a radical on the cofactor cobalamin, and class III enzymes form a stable glycyl radical with the help of an iron-sulfur protein and S-adenosyl methionine [11]. Though the metal cofactors used for the catalytic activity are different; only in class II, the metal cofactor may interact directly with the active site cysteine, whereas in class I and III, a stable protein radical is generated on a separate site, and the radical state is transferred to the active site by a radical transfer pathway consisting of a chain of hydrogen bonded amino acid residues [10].

Interestingly, in the microbial world, several organisms possess the genes encoding different RNRs that present different structures and allosteric regulation [27]. It might be an advantage for a pathogen to have opportunity to switch between classes, for example in response to the environment regarding to the existence of oxygen. Though, it is more difficult to understand why several bacteria contain both class I and II, and some even have genes for all three classes [4]. Higher organisms, only have class I, however, in mammalian cells different isoforms of small subunit has been found [28].

Table 1. Overview of the various classes of RNR.

	Class Ia	Ib	II	III
Occurrence	Eukaryotes/*E.coli*	Prokaryotes	Prokaryotes	Prokaryotes
Environment & metabolism	aerobic	aerobic	aerobic/anaerobic	strictly aerobic
Cofactor	Fe-O-Fe	Fe-O-Fe	cobalamin	[4Fe-4S]
Substrate	NDP	NDP	NDP/NTP	NTP
Reductant	Trx/Grx	NrdH-redoxin/Grx	Trx	Formate
Radicals involved	Tyrosyl	Tyrosyl	adenosylcobalamin	adenosyl-methionine, glycyl
Prototype	*E.coli*	*Salmonella Typhimurium*	*Lactobacillus Leichmannia*	*E.coli*

Of the three main classes of ribonucleotide reductase, class Ia, hereafter designated as RNR, is the most intensively studied, and is the basis for work described in this thesis.

1.2.2 Overall Structure of Class I

The mammalian enzyme, entitled in class I, consists of two non-identical subunits: R1 (α) and R2 (β). The R2 subunit is a homodimer (2 × 45 kDa) contains a tyrosyl free radical and an iron center in each polypeptide [11]. The R1 subunit contains the active site, and sites for allosteric effectors; R1 complex formation mainly comes from the allosteric effectors and in the absence of nucleotide effectors, the mammalian R1 is a 90 kDa monomer [29, 30]. Binding of nucleotide effectors to the specificity site induces the formation of R1 dimer that interacts with the R2, forming an active $\alpha_2\beta_2$ complex. The $\alpha_6\beta_2$ octamer complex is established as the major form of mammalian RNR *in vivo* [31].

Transcription of both R1 and R2 genes occurs exclusively during the S-phase [32, 33], but due to longer half-life, the level of R1 was thought to be constant throughout the cell cycle and always in excess of the level of R2 [32]. The cell cycle dependent activity of RNR is regulated mainly by the synthesis and degradation of the R2

protein. Recently the concentration of R1 protein in logarithmically growing and S-phase of mouse fibroblast Balb/3T3 cells has been calculated to be around 48 μg/ml but reduces to 5.3 μg/ml in resting cells [34]. When R2 is not available, R1 associates with p53R2 (a homologue of R2) to form active RNR that supplies quiescent cells with dNTPs for repair of damaged DNA and mithochondrial DNA synthesis [35, 36]. P53R2 was proposed to be induced by p53 after DNA damage to provide deoxynucleotides for DNA repair [28].

There have been conflicting results regarding the localization of RNR. R1 and R2 were claimed to move to the nucleus during S-phase for DNA replication [37, 38]. This idea was doubted with the early immunohistochemical studies [39]. Translocation of p53R2 from cytosol to the nucleus was also reported as a response to DNA damage to deliver deoxynucleotides at the site of their use [28, 38]. However, recent investigations with different methods revealed that all three subunits are localized in the cytosol; suggesting that the produced dNTPs by the enzyme diffuse into the nucleus or are transported into mitochondria [40].

1.2.3 R1 Subunit

R1 protein is the most dynamic part of RNR containing not only substrate binding site and the active site, but also two functionally different allosteric sites, termed the specificity, and overall activity sites. This unique allosteric regulation controls RNR in such a way that binding of triphosphate nucleotides regulates the substrate specificity and overall enzymatic activity [17]. This regulation is needed to ensure that the intracellular levels of the four DNA building blocks are balanced and not generally too high.

The specificity site effectors also induce the formation of R1 dimer and enzymatically active complex with R2 [18, 41, 42]. The strength of the interactions can be modulated by substrates, reducing agents, the pH-value and salt concentrations. Crystallization of R1 together with different allosteric effectors and substrates showed the conformational change in the R1 which influences binding of the correct substrate to the second R1 polypeptide [22]. The general allosteric effector site, located far from the active site, appears to regulate subunit interactions within the holoenzyme as well.

Recent studies proposed a third hypothetical allosteric site, the hexamerization site (H-site), occupied by ATP inducing the formation of R1 hexamers that can form a hyperactive complex together with the R2 [18, 43]. More evaluations revealed that the $\alpha_6\beta_2$ is the major complex form of RNR *in vivo* [31], which could be either in a hyperactive form in the presence of ATP, or in an inactive form in the presence of dATP.

In contrast to most other known thiol redox proteins, the redox-active cysteines of R1 are not located sequentially close to each other (that is, with two or four intervening residues) but are separated by hundreds of residues. The cysteines that have been proposed to be directly involved in the substrate reduction (C225 and C462 in *E.coli*; C218 and C444 in human/mouse R1), are instead held close by their location on two adjacent β-strand [44-46]. All three cysteines in the active site are conserved in the sequence of all class I R1 [10].

Besides the active site, two additional cysteines located close to the carboxyl end (C754 and C759 in *E.coli*; C787 and C790 in human/mouse R1) are involved in the reactivation of the active site. The last 24 residues at the carboxyl end, which includes these two cysteines, are not visible in the X-ray crystallography electron density maps [44]. This agrees with the hypothesis that the carboxyl end of R1 acts as a flexible shuttle of reducing equivalents from the surface of the molecule to the active site [44]. The first disulfide bond formed in the active site is subsequently transposed to the C-terminal disulfide bond, which is then reduced by an external thiol reduction system [47] (*see below*).

1.2.4 R2 Subunit

R2 subunit is the smaller component of RNR and serves as the activating part of the enzyme [10]. Deeply buried within the protein is the iron centre that is responsible for generating the radical on the tyrosyl residue (Tyr122 in *E.coli*; Tyr177 in mouse) which is essential for the catalytic reaction [48]. Organic radicals are highly reactive and can not be allowed freely in the cells, the radical reaction must therefore not be initiated until the substrate is bound at the active site of the R1 protein. The tyrosyl radical is buried about 10 Å inside the R2 protein and is stable *in vitro* for weeks at 4ºC [49, 50].

The tyrosyl radical cannot participate directly in the reaction taking place at the catalytic site of R1, but indirectly generates a second transient radical on R1. All class I RNRs have a net of conserved residues between the active site of R1 and the tyrosyl radical of R2; the hydrogen-bonded conserved residues have been proposed to function as a pathway for radical transfer between the tyrosyl in R2 and the cysteine residue in R1 [51-53]. This has been established by mutational studies of the residues conserved in the pathway [54]. The radical-harbouring tyrosine residue is suggested to be reduced and re-oxidized in each turnover [55].

The R2 structure is an all-helical protein structure and the di-iron site is located in a four helix bundle [50]. In the case of the mouse R2 structure the di-iron site is more accessible than for the bacterial R2s and only one of the two irons are present in the active site of either structure [56]. The C terminus of R2 is important for binding to R1 and peptides corresponding to the C terminus of R2 inhibit the formation of the holoenzyme complex. These interactions are species specific. The binding of carboxyl end peptides of R2 to R1 is of interest because of its possibilities as a basis for the design of species-specific antiproliferative drugs.

The cell cycle dependent activity of RNR is controled by the synthesis and degradation of the R2 protein, which even at its maximum in the S-phase, does not exceed 0.5-0.8 μM [57, 58]. Its synthesis starts in early S-phase, and then the R2 slowly accumulates in the cell up to late mitosis when it is rapidly degraded [59]. R2 contains a KEN-box at N-terminal (positions 30-32), which binds Cdh1-anaphase-promoting complex formed during mitosis, leading to ubiquitination and proteolysis [60].

1.2.5 P53R2

P53R2, a homologue of R2, has been identified in human and mouse cells [28] created a link between the synthesis of dNTPs and the tumor suppressor, p53. P53R2 can form an active RNR with R1 [35]. The p53R2 gene is located on chromosome 8 while the R2 gene is located on chromosome 2 in human [28]. Several of the functional domains present in R2 are also found in p53R2, including iron ligands, tyrosine radical, pathway for radical transfer from small to large subunit, and C-terminal sequence that binds R1 [28, 61].

The major difference between R2 and p53R2 is that the latter lacks 33 amino acid residues in the N terminus (KEN-box) required for degradation during mitosis [62]. It has been shown that the kinetic activity of p53R2-containing RNR is about 20-50% lower than R2-containing RNR. The difference may reflect the fact that although p53R2 binds to the same C-terminal heptapeptide in R1 as R2, the binding affinity for R1 is 4.76-fold lower [63, 64].

Before the discovery of p53R2, it was believed that in postmitotic cells, which are completely devoid of R2, the salvage of deoxynucleosides provides dNTPs for DNA repair and replication of mitochondrial DNA. Although, p53R2 was originally considered as an element of the DNA damage response, recently it was found in quiescent cells in the absence of DNA damage, at 30-fold lower than R2 level in S-phase [34]. Similar to the R1-R2 complex in cycling cells, outside the S-phase the only active form of RNR is R1-p53R2. It is reported that in quiescent human fibroblasts this complex catalyzes ribonucleotide reduction at 2-3% of the rate of R1-R2 [65], which together with the salvage pathway [6], provides dNTPs for DNA repair and mitochondrial DNA replication.

It is now established that *in vivo* in conjunction with R1, p53R2 is required for mitochondrial DNA synthesis in differentiated tissues. Mutant mice lacking p53R2 grow apparently normally up to 6 weeks but then die from glomerular injury and kidney failure [66, 67]. Moreover, it is shown that children with severe mitochondrial depletions carry functionally important mutations in p53R2 [68], which demonstrates its role for mitochondrial DNA replication.

The existence of both the small subunit R2 and p53R2, with their striking similarity at the sequence level, indicates the importance of maintaining equilibrium between DNA repair, DNA replication and cell viability. Elucidation of the p53R2 interactions with other proteins will provide important information which can be applied in the clinical development of anticancer drugs.

1.3 EXTERNAL ELECTRON DONORS

The two major oxidoreductase systems of the cells are the thioredoxin (Trx) and the glutaredoxin (Grx) systems [69, 70], which both utilize reducing equivalents from NADPH. Trx was originally isolated in 1964 as a small heat-stable redox protein on its ability to reduce the disulfide formed in *E.coli* RNR during its catalytic cycle [71]. Normal growth of an *E.coli* mutant lacking Trx, led to the discovery of Grx as glutathione-dependent reductase to assist RNR [72].

Oxidized Trx is reduced to its thiol form by NADPH via the action of the thioredoxin reductase (TrxR). Oxidized Grx utilizes the reducing power of glutathione (GSH), which, in turn, is reduced by NADPH and glutathione reductase (GR) (Fig. 3) [14, 73]. Although the two systems share a number of similarities, and display some overlapping and complementary activities, they do not act as simple backup of each other and have a series of specific differences and activities [73].

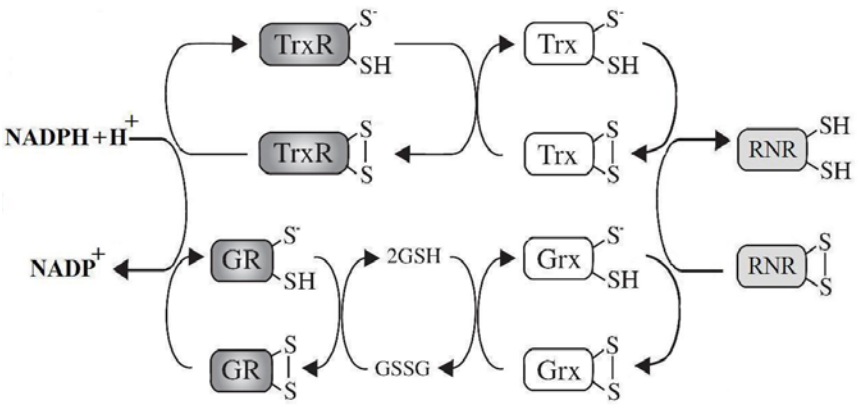

Figure 3. The thioredoxin and glutaredoxin systems from *E.coli.*

1.3.1 The Thioredoxin System

The thioredoxin system composed of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is the major disulfide reducing enzyme in all cells responsible for maintaining the intracellular redox milieu. Separate Trx and TrxR enzymes operate in the cytosol and the mitochondria [69, 74, 75]. Trx and TrxR have been reported to be overexpressed in many aggressive tumor cells in which the proliferation is crucially dependent on a constant deoxyribonucleotide supply [76, 77].

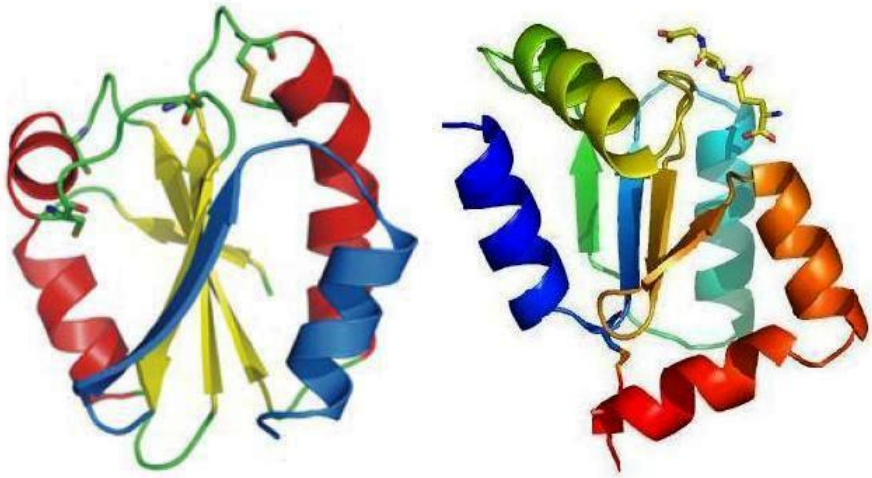

Figure 4. (*Left*) The structure of oxidized human thioredoxin-1 (PDB Code: 1ERT), and (*Right*) human glutaredoxin-2 with glutathione (PDB Code: 2FLS).

1.3.1.1 Thioredoxin

Thioredoxin is a ubiquitous enzyme present in various species and tissues [14]. Trxs contain two redox active cysteines in a conserved Cys-Gly-Pro-Cys active site motif that during the catalysis, cycle between a dithiol and an intramolecular disulfide. Mammalian cells contain two distinct Trxs, termed Trx1 and Trx2. Trx1 is mainly a cytosolic protein, though, it can be translocated to the nucleus at certain signaling events [78], or exported from the cell.

Extracellularly it is found both as a full-length Trx1 and a shorter truncated form, called Trx80. These two extracellular forms can act as cytokine or chemokine [79-81]. Trx1 has 105 amino acid residues and is a compact globular protein, composed of a single twisted β-pleated sheet containing five strands which is flanked by four α helices on the external surface [82, 83].

Trx2 is a mitochondrial protein with an N-terminal extension of 60 amino acids as the mitochondrial translocation signal [84]. Both isoenzymes show 35% sequence identity and reveal similar catalytic properties *in vitro* [84]. Thioredoxins possess essential roles in cell survival and lack of either Trx1 or Trx2 is embryonically lethal [85, 86].

1.3.1.2 Thioredoxin Reductase

Thioredoxin reductase (TrxR) is a flavoprotein catalyzes the NADPH-dependent reduction of thioredoxin. It belongs to the family of pyridine nucleotide-disulfide oxidoreductases which catalyze the electron transfer from pyridine nucleotides to disulfides [87]. TrxR is a ubiquitous enzyme present in all living cells. However, the level of TrxR in tumor cells is often 10-fold or even greater than in normal tissues, and tumor survival and proliferation seem to be crucially dependent on an active thioredoxin system, which makes it a potential target for anticancer drugs [88].

Mammalian TrxR is composed of two identical subunits of 55 kDa which are organized in a so called "head-to-tail" pattern. Each subunit contains a flavine adenine dinucleotide (FAD)-binding domain, an NADPH-binding domain, and an interface domain [89-91]. In contrast to the TrxRs in lower organisms, mammalian TrxR is a large selenoprotein and contains a conserved C-terminal active site sequence Gly-Cys-Sec-Gly-COOH together with an N-terminal redox active disulfide [92, 93]. More detail of catalytic mechanism of mammalian enzyme has been reported by crystallization of the recombinantly expressed rat TrxR [91].

Besides Trx, mammalian enzymes have a broad spectrum of substrates, ranging from small molecules such as selenite, lipid hydroperoxides, ebselen, and dehydroascorbate to proteins like protein disulfide isomerase or glutathione peroxidase, etc [69, 94]. Most of these substrates are involved in cellular redox regulation; therefore, TrxR plays a central role in maintaining the redox homeostasis directly or with Trx as well.

Three mammalian TrxR isoenzymes have been described: cytosolic TrxR1 [95, 96], mitochondrial TrxR2 [97, 98], and the testis-specific TGR [99]. The third, in contrast to TrxR1 and TrxR2 can reduce glutathione disulfide in addition to Trx, and therefore has been named TGR, indicating its thioredoxin/glutathione reductase activity.

1.3.2 The Glutaredoxin System

The glutaredoxin system composed of glutathione (GSH), glutaredoxin (Grx), glutathione reductase (GR), and NADPH [70, 100].

1.3.2.1 Glutathione

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is the major non-enzymatic antioxidant and the most abundant thiol compound in the cell [101]. GSH is synthesized in the cytosol via the action of two ATP-dependent enzymes, γ-glutamyl-cysteine synthetase and glutathione synthetase, from its constitutive amino acids [102-104]. The exceptional peptidic γ -linkage is thought to protect the tripeptide from degradation by aminopeptidases.

Upon oxidation, two molecules of GSH form glutathione disulfide (GSSG), sometimes referred to as "oxidized glutathione". In most organisms, GSH is kept in the reduced form at the expense of NADPH with the help of the dimeric flavoenzyme glutathine reductase.

$$2\ GSH + R\text{-}S\text{-}S\text{-}R \longrightarrow 2\ R\text{-}SH + GSSG$$

$$GSSG + NADPH + H^{+} \longrightarrow 2\ GSH + NADP^{+}$$

GSH together with its oxidized counterparts, GSSG and protein-SG mixed disulfides, constitute the major redox buffer of the cell [105]. It is present in millimolar concentrations in the cell and the major determinant of the cellular redox state which is correlated with the biological status of the cell [106]. A significant amount of GSH (up to 15%) is bound to the proteins in the cell [107]. GSH regulates the activity of numerous physiological processes such as cell growth, differentiation, and metabolism, etc by S-glutathionylation [108]. The S-glutathionylation of proteins can protect them from irreversible oxidation of cysteine residues [109, 110]. This reaction can be affected by the redox potential of the cells as S-glutathionylation can happen via a thiol/disulfide exchange reaction between Cys residues and GSSG. However, it may occur under normal conditions with oxidation of the protein thiol or GSH with formation of a mixed disulfide [111].

1.3.2.2 Glutaredoxin

Glutaredoxins (Grx) are defined by their ability to bind and utilize GSH as substrate. Structurally, they belong to the Trx-fold family of proteins. Two groups of Grxs have been characterized; the early discovered dithiol Grxs containing the Cys-Pro-Tyr-Cys

motif as active site, and the monothiol group lacking the C-terminal thiol in Cys-Gly-Phe-Ser active site [70]. Human cells contain two dithiol Grxs: the cytosolic Grx1, and the mainly mitochondrial Grx2a. Grx2a with the active site Cys-Ser-Tyr-Cys is also reduced by TrxR [112]. Some cancer cells and testicular cells express two additional cytosolic/nuclear isoforms (Grx2b and Grx2c) [113].

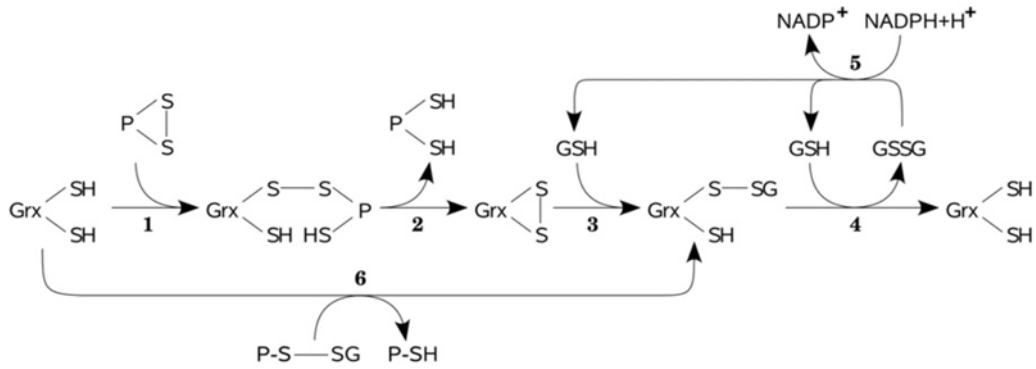

Figure 5. Reaction mechanisms of glutaredoxins. Grxs catalyze the reduction of protein disulfides utilizing both of their active site cysteinyl residues (reactions 1–4). Disulfides between GSH and proteins or low molecular weight compounds are reduced in the monothiol mechanism that requires only the more N-terminal active site cysteinyl residue (6 & 4). In either case, GSH disulfide formed in the reaction is reduced by glutathione reductase at the expense of NADPH (5). *(Adapted from Biochim Biophys Acta, 2008, 1780:1304-17)*

In contrast to Trxs, beside the dithiol mechanism for disulfide reduction, Grxs may also act via a monothiol mechanism in which only the N-terminal Cys of the active site is required; in fact most dithiol Grxs characterized have a strong preference for GSH-mixed disulfides as substrates [114, 115]. In the dithiol mechanism the disulfide bond in the target protein is reduced at the expense of two electrons from active site cysteinyl thiolates of Grx, whereas in the monothiol mechanism the protein-GSH disulfide is attacked by the more N-terminal Cys residue generating a Grx-GSH mixed disulfide and releasing the target protein. A second GSH is needed for reduction of the disulfide formed between Grx and GSH which is the rate limiting step of this mechanism (Fig. 5) [116].

With the second mechanism, disulfides between GSH and proteins or low molecular weight compounds are reduced. The ability to use GSH and the high

specificity for GSH-mixed disulfides, distinguishes Grx from Trx. Grxs catalyze both the formation and the reduction of mixed disulfides between GSH and thiols of proteins [117]. The deglutathionylase activity is a prominent role in homeostasis of protein sulfhydryl groups, for protection under oxidative stress and regulation, whereby reversible glutathionylation acts as a redox-mediated signal transduction mechanism.

1.3.2.3 Glutathione Reductase

Glutathione reductase (GR) belongs to the pyridine nucleotide-disulfide oxidoreductases family of proteins which also contains thioredoxin reductase. The main function of this enzyme is to maintain the supply of reduced GSH of the cell. However, mild alteration in the GSH/GSSG ratio in GR null mutants of *E.coli* and *Saccaromyces cerevisiae*, indicate alternative reducing pathways [118].

The human GR has a molecular mass of 102 kDa and is composed of two identical subunits which are organized “head-to-tail” in a similar fashion as TrxR. Each subunit consists of three domains including an NADPH-binding domain, an FAD-binding domain, and an interface domain [119]. To reduce GSSG, two electrons are transferred from the binding of NADPH to the flavin moiety, which reduce the active site cysteine disulfide located in the same subunit. After a disulfide/dithiol exchange reaction, electrons are transferred to GSSG to reduce it to GSH [120]. GR is detected in both cytosol and mitochondria in mammalian cells. The two proteins have indistinguishable biochemical properties and are encoded by a single gene [121].

1.3.3 Characterization of Electron Donors of RNR

Ribonucleotide reductase is the key enzyme for reduction of all four ribonucleotides to the corresponding deoxyribonucleotides for DNA synthesis. The enzyme uses a free radical mechanism plus –SH groups that are oxidized to a disulfide during reduction of the 2′–OH of the ribose moiety. Turnover of the enzyme requires reduction by a dithiol [13].

Thioredoxin and glutaredoxin are small ubiquitous proteins containing an active site with redox-active cysteine residues, active in electron transfer via the reversible oxidation of two –SH groups to a disulfide bridge. Trx was originally isolated as the natural hydrogen donor for *E.coli* RNR [71, 122]. The thioredoxin system was for a decade, the only known natural hydrogen donor for RNR, when an alternative hydrogen transport pathway from NADPH, dependent on reduced glutathione, was discovered [72]. The viability of $Trx^{(-)}$ mutants of *E. coli* gave the clue to the identification of a novel cofactor, Grx1, as an efficient substitute of Trx for RNR. Grx1 and Trx1 of *E. coli* have similar V_{max} but the K_m for Grx1 is 10-fold lower (0.15 μM compared to 1.2 μM of Trx1) [123]. These suggest that in *E. coli,* Grx1 is the pivotal electron donor for the reduction of RNR.

The isolation of a viable *E. coli* double mutant, lacking both Trx1 and Grxl led to the postulation of the existence of two more glutaredoxins termed: Grx2 and Grx3 [124, 125]; which only Grx3 was able to serve as a weak hydrogen donor for RNR. As a hydrogen donor for *E. coli* RNR, Grx3 shows approximately the same K_m-value (0.35 μM) as Grxl, whereas its V_{max}-value is only 5% of that of Grxl. The combination of the Grx3 hydrogen donor activity and a 25-fold induction of RNR activity in a $Trx^{(-)}$ $Grx^{(-)}$ double mutant provides an explanation for its viability and deoxyribonudeotide biosynthesis.

Moreover, the viability of a triple Trx1, Grx1, and Grx3 mutant, indicated the presence of another alternative protein capable of reducing RNR *in vivo*, leading to the identification of a second thioredoxin. This enzyme termed Trx2 can also reduce RNR *in vitro* but with slower kinetics compare to Trx1 [126].

E.coli contains three different classes of RNR: class Ia (NrdAB) and Ib (NrdEF), plus anaerobic class III (NrdDG). In place of Trx or Grx used by class Ia, the class Ib uses NrdH-redoxin (NrdH) protein as electron transporter (K_m = 1.2-1.6 μM) [127].

The absence of GSH in some bacterium raised the question of the identity for NrdH [128]; this glutaredoxin-like enzyme is reduced by thioredoxin reductase and not by glutathione. Though *E.coli* Trx1 is inactive with class Ib, Grx1 is active with slightly slower kinetics than those of NrdH (K_m = 1.2-5 μM). The weak activity of Grx1 to reduce NrdEF, when overexpressed, is reminiscent of the ability of the weakly active Grx3 to reduce NrdAB, when the later is overexpressed [129]. NrdH can also reduce class Ia with a high K_m of 4.2 μM [127].

The field was expanded even more by discovery of a family of monothiol Grxs, such as Grx4 in *E.coli* [130, 131], which is outside the scope of this investigation.

No such detailed analyses of RNR reductants exist for mammalian cells, and despite of its importance, the identity of the mammalian RNR external thiol reductant system has been remained undetermined. Most of what we know has been done by Luthman *et al* in 1980s on calf thymus RNR [132, 133], and so far most of experiments with RNR have been performed with DTT as a chemical reductant.

1.4 RNR INHIBITORS

Ribonucleotide reductase as an enzyme that plays a critical role in the mechanism of DNA replication, as well as an indirect role in regulating other enzymes in the DNA synthetic pathway, represents an important target for the design and synthesis of antiviral and cancer chemotherapeutic agents [134]. In tumor cells the need of dNTPs for proliferation is high and for example the apparent activity level of RNR in rapidly growing hepatoma cells is ~100-fold more than that of normal liver cells [135]. As such, RNR has long been considered as an excellent target for cancer chemotherapy [136], because small decrease in the intracellular concentrations of the dNTPs causes large decrease in DNA synthesis and cell proliferation [137].

The RNR inhibitors can be classified in two main categories according to their target and mechanism of action:

a) *Protein inactivators:*

- R1 inhibitors that interact with R1 to inhibit the active site (inactivators of sulfhydryl groups) or nucleoside analogues (including substrate analogues and allosteric effector analogues).
- R2 inhibitors which destroy the essential di-iron tyrosyl radical center (radical scavengers or iron chelators).
- Polymerization inhibitors that prevent the formation of RNR holoenzyme, like the oligopeptides corresponding to the C terminus of R2 [138].

b) *Gene expression regulators,* which regulate the RNR gene expression with subunit specificity, like R1 or R2 antisense inhibitors.

Efforts for new RNR inhibitors have been made in basic and translational research. In recent years, several RNR inhibitors have entered clinical trial or application. In addition, the discovery of p53R2 raises the interest to develop subunit-specific RNR inhibitors for cancer treatment. However, there are few RNR inhibitors currently in clinical use, like the radical scavenger hydroxyurea that has been marketed for cancer therapy for decades [139], or the substrate analogue gemcitabine [140, 141]; and others are being evaluated in clinical trials.

1.4.1 Motexafin Gadolinum (MGd)

Motexafin gadolinium, also known as Xcytrin® (Pharmacyclics, Inc.), belongs to a class of compounds known as texaphyrins with biological activity as an anticancer agent. Texaphyrins are synthetic expanded porphyrins capable of forming complexes with large metal cations such as lanthanides. MGd is the name given to a specific texaphyrin in complex with gadolinium.

Although, the mechanism of action of this compound has not been fully elucidated, evidence has suggested that MGd generates reactive oxygen species, resulting in disruption of the cellular metabolism and apoptosis [142-146]. A standard biochemical reduction potentials of -41 mV for MGd indicates the strong electron affinity of this compound [142], and in the presence of oxygen such electron transfer results in superoxide formation and regenerates the texaphyrin. Superoxide anion, in turn, will disproportionate under biological conditions to form oxygen and hydrogen peroxide [147]. It has been shown that MGd catalyzes the oxidation of critical protein thiols and several intracellular reducing metabolites such as glutathione, ascorbate and NADPH. Moreover, MGd disrupts a number of key enzymes important in cellular processes, leading to an increase in intracellular free zinc and metallothionein production [148, 149].

On the other hand, redox mechanisms have been shown to be important in malignant cell survival and that these may be modified for the treatment of malignancies. Since MGd has a mode of action based on redox cycling, it may present a novel approach for cancer therapy. It is currently being evaluated in many human clinical trials as monotherapy and in combination with radiation and/or chemotherapy [150-155], especially in combination with radiotherapy for the treatment of brain metastases [144, 156]. The combination of protein and metabolite oxidation and generation of ROS, induces apoptosis and alters the threshold for cytotoxicity of many commonly used chemotherapy agents.

Both animal and human trials have shown that MGd selectively accumulates in malignant cells; this advantage together with the presence of gadolinium in MGd, allows for visualization of the tumor by magnetic resonance imaging (MRI) without the use of contrast agents [144, 157]. However, MGd is not suitable as a contrast

agent in intraoperative MRI for the detection of remaining tumor tissue during surgery [158].

The radiation sensitizing properties of MGd have been investigated in a number of clinical trials involving patients with brain metastases. These studies clearly show that MGd is detectable in magnetic resonance images many days following administration. The aim of this experimental study was to test whether MGd could serve as an efficient intraoperative contrast agent avoiding problems that arise with surgically induced intracranial enhancement. A new study provides initial evidence that MGd may enhance MRI of vessel wall for the characterization of plaque in deep-seated arteries [159].

Overall, several studies have elucidated a number of properties that makes MGd particularly suited for the treatment of cancer. In summary:

- MGd selectively targets tumor cells which have increased rates of metabolism.
- It remains inside cells for days [156].
- It disrupts energy production and inhibits cellular repair by stimulating the production of ROS such as free radicals [160].
- It is detectable by magnetic resonance imaging [144].
- It does not accumulate in normal brain protected by an intact blood–brain barrier [158].

MGd is actively being assessed in clinical trials, and is likely to play an increasing role in the management of cerebral metastases in future. As the redox perturbations are a component of many diseases, it is believed that redox active drugs offer a new approach to the treatment of cancer and some other disorders. The oxidation of vicinal thiols such as thioredoxin might be the most important process under intracellular conditions.

2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

- To characterize the reactivity of MGd with thioredoxin reductase and thioredoxin, as well as the interaction with ribonucleotide reductase.

- To elucidate the mechanism of inhibition of ribonucleotide reductase with MGd.

- To evaluate the kinetics of the thioredoxin and glutaredoxin systems as electron donors for mammalian ribonucleotide reductase.

- To identify isoforms of mouse Grx2, and further characterize the different isoforms with respect to subcellular localization, expression pattern, and enzymatic activity.

2.2 METHODS

Different techniques applied in this thesis are presented in the respective individual papers. In this section the RNR assay which is the base of this thesis study, and fluorescence correlation spectroscopy (FCS) as a rather new technique in biological research, are discussed in more details.

2.2.1 Determination of RNR Activity (RNR Assay)

The most common assay to test the enzyme activity of RNR, which we applied in our studies, is monitoring the production of deoxynucleotides using radioactive labeled substrate [161, 162].

The active RNR was reconstituted by mixing recombinant R1 and R2 subunits. Reducing equivalents were provided by DTT, Trx system, or Grx system in separate experiments. The Trx system was composed of Trx, 0.1 μM TrxR, and 1 mM NADPH. When the Grx system was used, the samples contained 0.1 μM GR, 1 mM NADPH and different amounts of GSH (generally 4 mM). The reaction was initiated by adding reaction mixture containing 40 mM Tris-HCl buffer, pH 7.6, 2 mM ATP, 10 mM $MgCl_2$, 200 mM KCl, 200 μM $FeCl_3$ and 0.5 mM [^{3}H]CDP (~20,000 cpm/nmol) in a final volume of 50 μl.

Samples were incubated at 37ºC usually for 30 min; thereafter, reaction was terminated by addition of 1 M $HClO_4$. A known amount of carrier dCMP (approximately 0.7 μmoles) was added, and the solutions were boiled for 10 min to break pyrophosphate bonds. After neutralizing the samples with 4 M KOH (Phenol red), and hydrolysis to dCMP, the precipitation was removed by centrifugation and the supernatant was applied on manually packed 7-ml Dowex-50 columns (ion-exchange chromatography). Columns were washed through over night with 106 ml acetic acid (0.2 M), and were eluted with 30 ml acetic acid (0.2 M) the following day. The amount of [^{3}H]dCMP radioactivity in elutions was quantified by liquid scintillation counting, and the activity was calculated as nmol dCDP produced per time of incubation corrected for the yield of the columns. The correction factor ranged between 70 and 90%, obtained from the known amount of carrier added and the measured absorbance of the dCMP fraction (at 280 nm).

Monitoring the consumption of NADPH (the ultimate reducing equivalents) in real time is the other alternative method which could be applied. The reaction mixture is the same as [^{3}H]CDP assay, except that radioactive labeled substrate is changed to cold CDP. After addition of CDP to the samples containing RNR and the reaction buffer, NADPH consumption was monitored at 340 nm by a spectrophotometer at room temperature. Reduction of RNR is measured as the decrease of NADPH absorbance over time using molar extinction coefficient of 6,200 $M^{-1}cm^{-1}$ in calculations. Although, this method is much faster, comparing to the rather time-consuming [^{3}H]CDP assay, as an indirect assay it is less accurate. In addition, applying cell extract with this method is tricky.

The assay of RNR activity in the crude cell extracts is complicated by the presence of competing activities that rapidly deplete the substrate; either hydrolyzing enzymes that dephosphorylate, or kinases that phosphorylate the ribonucleoside diphosphate substrate [163]. Different efforts have been applied to overcome this problem, such as: using a non-cleavable ATP analogue, which is effective at minimizing substrate diversion [163], or conducting assays in permeable cells [164, 165]. Although, the latter technique is argued not to be able to exclude incorporation of labeled ribonucleotide into RNA [166], unless enough NaOH is added.

Another method has been also introduced [167] to detect RNR activity in cell extracts where production of radioactive-labeled deoxyribonucleotides by RNR is calculated via coupling to the DNA polymerase reaction, and is enhanced by using RNase to degrade endogenous RNA.

2.2.2 Fluorescence Correlation Spectroscopy (FCS)

FCS is a powerful tool for examining molecular interactions by measuring the diffusion time which depends on the molecular weight of the compound/complex [168]. It has the ability to quantify the concentrations of both free and bound ligand, which is a prerequisite to the determination of potency and affinity of the ligands. The advantage of this technique is that there is no need for separating unbound ligands from the bound ones to calculate the receptor bound, and free ligand fractions. FCS is also a sensitive single-molecule detection assay to study fast dynamic interaction processes in living cells.

The FCS technique was introduced in the early 1970s [169, 170], and improved over time, concerning both the theoretical basis and developing of the instrument. Recently it has become applicable to bioscience because of a substantial increase in sensitivity [168] allowing single-molecule analysis. In this technique, the fluorescence of single dye-labeled molecules excited by a sharply focused laser beam is observed (Fig. 6). From the fluorescence intensity fluctuations due to variations in numbers of molecules in the laser volume element of observation, the average number of molecules can be directly obtained using the intensity correlation function. Furthermore, from the characteristic correlation times, dynamic processes such as Brownian motion can be analyzed.

The tiny laser volume elements (0.2 fl) in which the measurements are performed, make it possible to evaluate molecular interactions not only in solution, but also at the cell membrane [171]. With FCS one can analyze a mixture of multiple components possessing different molecular weights and different diffusion times, respectively.

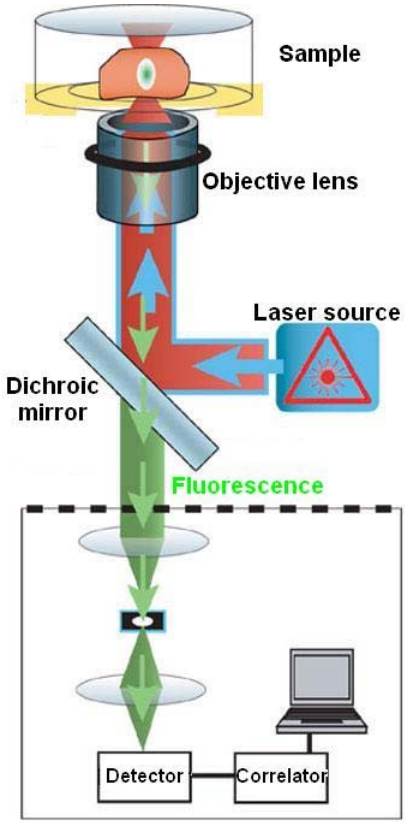

Figure 6. Schematic design of FCS instrumentation: A parallel laser beam for one- or two-photon excitation is coupled into the microscope (red beam path) via a dichroic mirror epi-illuminating the objective lens. A small, diffraction-limited focal spot serves as the FCS observation volume in which fluorescence is generated. Fluorescence light is collected via the same objective lens, passed through the dichroic mirror (green fluorescence path), and finally projected onto a high-sensitivity detector (single-photon counting module). The digital photon signal is sent to a hardware correlator. Respective correlation curves are displayed and stored on a computer for subsequent analysis. (*Adapted from Nature Methods, 2007, 4:963-73*)

2.3 RESULTS AND DISCUSSIONS

2.3.1 Paper I

Motexafin gadolinium, a tumor-selective drug targeting thioredoxin reductase and ribonucleotide reductase. *(JBC 2006; 281: 10691-7)*

Adding increasing amounts of MGd resulted in a saturable oxidation of NADPH in the presence of either rat or *E.coli* TrxR, indicating that MGd is a substrate for this enzyme. The addition of TrxR dramatically accelerated NADPH oxidation and only in the presence of oxygen, significant NADPH oxidation occurred which led to the production of reactive oxygen species. This reaction required the active enzyme, since in the presence of DNCB (an irreversible inhibitor of human TrxR) no reaction with MGd was shown, but addition of fresh TrxR to this sample could cause NADPH oxidation. Moreover, a mutant TrxR with the Sec498Cys mutation did not show any reactivity with MGd.

To investigate the binding of MGd to TrxR, we used a PD-10 Sephadex column equilibrated with 40 μM MGd; enzyme incubated with MGd was added to the column and a chromatogram was generated. The co-elution of MGd with TrxR showed the binding between MGd and the enzyme.

The reaction between MGd and Trx was very slow, and there was no evidence of complex formation between MGd and Trx. The inhibitory effect of MGd on protein disulfide reductase activity of the Trx system was evaluated by insulin disulfide reduction assay with varying concentrations of MGd. The inhibitory effect with the IC_{50} of around 50 μM was observed. Using different amounts of Trx with and without MGd revealed that it was a non-competitive inhibitor. We also assayed varying concentrations of NADPH and showed that MGd had a non-competitive inhibitory effect on NADPH.

Inhibition of TrxR was independent of Trx, since the strong inhibitory effect (with IC_{50} of 6 μM) was obtained even with DTNB, the artificial substrate of TrxR.

Effect of MGd on the RNR activity was studied using the Trx system as an electron donor. MGd inhibited mouse RNR with IC_{50} of 2 μM. The direct inhibition of RNR enzyme was proved by the inhibitory effect of MGd in the system which DTT was used as a source of reductant.

2.3.2 Paper II

Identification, expression pattern, and characterization of mouse Grx2 isoforms.
(ARS 2009; 11: 1-14)

We systematically screened for alternative transcript variants of mouse Grx2. We identified a total of six exons: three constitutive (II, III, and IV), two alternative first exons (exons Ia and Ic), and one single-cassette exon (exon IIIb) located between exons III and IV. The six exons give rise to five transcript variants that encode three protein isoforms: mitochondrial Grx2a, a cytosolic isoform that is homologous to the cytosolic/nuclear human Grx2c and present in specific cells of many tissues, and the testis-specific isoform Grx2d that is unique to mice.

The cellular localization of Grx2 isoforms in mouse tissues were analyzed by immunohistochemistry and confocal immunofluorescence microscopy. To analyze the properties of these isoforms, they were cloned, expressed in *E.coli,* and purified for further characterization.We showed that mouse Grx2c can form an iron/sulfur cluster-bridged dimer, and is enzymatically active as a monomer. This isoform can donate electrons to ribonucleotide reductase. Testicular cells lack mitochondrial Grx2a but contain cytosolic Grx2. Prominent immunostaining was detected in spermatogonia and spermatids.

These results provide evidence for additional functions of Grx2 in the cytosol, in cell proliferation, and in cellular differentiation.

2.3.3 Paper III

Mechanism of inhibition of ribonucleotide reductase with motexafin gadolinium (MGd). *(BBRC 2009; 379: 775-9)*

Different incubation times for RNR assay were used in an experiment with MGd. The percentage of inhibition increased by time of incubation from 30 to 65% for 10 and 60 min incubation, respectively, showing that the existence of MGd in the reaction could cause more inhibitory effect by time. The inhibition did not show any dependence on pre-incubation of the MGd with RNR before running the assay.

The sensitivity of RNR to reactive oxygen species was shown in an assay where RNR subunits were pre-incubated with different concentrations of hydrogen peroxide for one hour at room temperature. The activity of RNR was inhibited in a H_2O_2-dependent manner. Pre-incubation of each subunit separately with H_2O_2 and adding the non-treated subunit after one hour together with the reaction mixture, showed no difference in inhibition of RNR.

To test whether MGd can specifically inhibit one subunit, different concentrations of each subunit was checked with constant amount of the other subunit, with or without MGd. With constant R1, the inhibition with 10 μM MGd remained constant, while increasing the R1 concentration could compensate for the inhibitory effect.

The direct interaction between MGd and R1 was shown with fluorescence correlation spectroscopy (FCS). The diffusion times of the fluorophor MGd alone (10 μM), together with R1 (0.5 μM), or with both R1 and R2 were measured. High molecular complex formation between MGd and R1 was detected in the presence of R1. The R2 did not bind to the drug by itself, but could compete with MGd for binding to R1, and thereby inhibited binding of MGd to R1. Binding between R1 and MGd was confirmed with gel filtration using a PD-10 Sephadex column equilibrated with 40 μM MGd. R1 loaded onto the column could replace Sephadex-bound MGd which was indicated by co-elution of R1 and MGd.

To investigate whether this binding could take place *in vivo*, we used confocal microscopy. HeLa cells were synchronized and fixed during S-phase. Both, R1 and R2 subunits of RNR localized in the cytoplasm. HeLa cells were treated over night with 50 μM MGd before synchronization/fixation, and co-localization of MGd with R1 was observed.

2.3.4 Paper IV

Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for mammalian S-phase ribonucleotide reductase. *(JBC 2009; In Press)*

In characterization of mammalian RNR, the highest activity was obtained with 4-10 mM DTT ($K_m = 2$ mM), and inhibition by higher concentrations of DTT was observed. Unlike the *E.coli* RNR, no increase in activity was obtained by R1 pre-reduced with DTT.

Enzyme activity was measured with varying concentrations of human Trx1 using excess TrxR and NADPH, or different amounts of Grx1 in combination with 4 mM GSH, NADPH and excess GR. Typical apparent K_m-values of 1.9 and 0.18 μM were obtained for Trx1 and Grx1, respectively. With the higher concentrations of R1, the Trx activity curve showed a tendency to sigmoidal pattern probably reflecting rate-limiting reduction by low Trx. Remarkably the apparent V_{max} and corresponding k_{cat} was much lower for Grx than that for Trx. However, the catalytic efficiency (k_{cat}/K_m) was similar ($2 \times 10^5\ M^{-1}s^{-1}$).

Grx2 had the same activity as Grx1 except a marginally higher K_m-value. Similar experiments with a Cys40Ser mutant of Grx2 unexpectedly showed full activity with RNR. This demonstrates that Grx operates with mouse RNR by a monothiol mixed disulfide mechanism in sharp contrast to Grx1 and *E.coli* RNR where a monothiol Grx1 mutant is inactive. Since Grx2 is a substrate for TrxR, we checked 0.1 μM TrxR with Grx2 in RNR assay. The activity was about 10-fold lower than that with GR and 4 mM GSH showing that dithiol Grx is an inefficient electron donor for mouse RNR. Moreover, contrary to Trx1, Grx1 did not show any activity above the activity with a low concentration of DTT, suggesting that dithiol Grx was not an electron donor.

The effect of GSH was investigated in RNR assays using different concentrations of GSH; samples contained 1 μM Grx1 plus excess GR and 1 mM NADPH. Results revealed that the apparent K_m-value for GSH was 3 mM. Thus GSH was necessary for RNR reduction with Grx; experiments performed with only high GSH as the only reducing source for RNR, confirmed that GSH *per se* was not active. Furthermore we measured the kinetics of Grx1 using 4 and 10 mM GSH, and a higher apparent k_{cat} with 10 mM GSH was observed.

2.4 CONCLUDING REMARKS

Paper I:

- MGd is a substrate for TrxR, and catalyzes the production of ROS from NADPH in the presence of oxygen. It also interacts directly with TrxR.
- MGd is a powerful inhibitor of Trx system, but does not react with Trx.
- MGd strongly inhibits RNR with either Trx system (IC_{50} of 2 μM) or DTT (IC_{50} of 6 μM) as electron donor, revealing its direct inhibitory effect on RNR.

Paper II:

- Three different isoforms of Grx2 exist in mouse (Grx2a, Grx2c, and Grx2d).
- Mitochondrial Grx2a is expressed in all mouse tissues except testis. Cytosolic Grx2c and Grx2d are expressed in testis; Grx2c is also present in specific cells.
- Mouse Grx2c has general Grx-activity and can reduce RNR, while Grx2d lacks enzymatic activity.

Paper III:

- Inhibition of RNR with MGd is dependent on the incubation time of the assay, consistent with the sensitivity of RNR to H_2O_2.
- MGd inhibits the large subunit of RNR, and forms complexes with it.
- In S-phase synchronized cells, both R1 and R2 subunits are localized in the cytoplasm, and MGd is co-localized with RNR.

Paper IV:

- With mammalian RNR, Trx1 and Grx1 have similar catalytic efficiency (k_{cat}/K_m). Grx1 displays a higher affinity ($K_m = 0.18$ μM) compared to Trx1 ($K_m = 1.9$ μM).
- Trx1 shows a higher apparent k_{cat} suggesting its major role in S-phase DNA replication.
- Grx activity is strongly dependent on GSH concentrations ($K_m = 3$ mM), and the activity of a monothiol Grx2 mutant, demonstrates a GSH-mixed disulfide mechanism for Grx, in contrast to the dithiol mechanism of Trx.

3 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Although mammalian RNRs belong to the same class as *E.coli*, the identity of their most efficient electron donor *in vivo* was unclear, and so far almost all of experiments have been done with DTT. Our analysis of the recombinant mouse RNR, revealed basic differences between mammalian and *E.coli* RNR, with respect of their electron donors, and demonstrates a GSH-mixed disulfide mechanism for Grx catalysis in contrast to the dithiol mechanism for Trx (Fig. 7) .

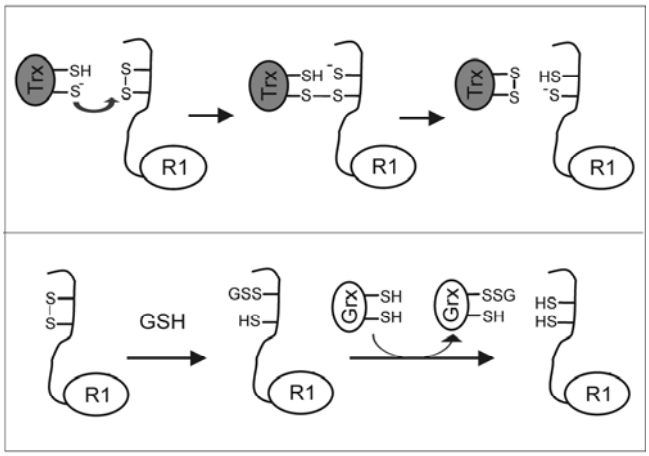

Figure 7. A schematic model showing the dithiol and monothiol reduction mechanisms of the mammalian RNR with thioredoxin and glutaredoxin.

Cycling cells require a large supply of dNTPs during S-phase, as the whole nuclear DNA should be replicated. Production of this amount of dNTP for replication forks in growing DNA chains during the limited period of S-phase may predominantly be supported by the Trx system. In contrast, small amounts of dNTPs are required for replication of mitochondrial DNA and continous repair of damaged DNA. The GSH-mixed disulfide mechanism for reduction of mammalian RNR could give an advantage to the Grx system with its higher affinity (K_m = 0.18 μM), since any Grx would act with glutathionylated R1; besides Trx is often not expressed in unstressed cells [69].

On the other hand, although Trx has been assumed as the main reductant in cells, it should operate by a ping-pong mechanism shuttling between both TrxR and RNR; obviously these bindings and dissociations, plus diffusion of the protein, lower the

turnover number unless somehow organized. In comparison, the Grx may be viewed as the more sophisticated system; although, Grxs are poor general disulfide reductants, they depend on a diffusible cofactor, GSH. GSH is a small molecule, and has a better chance for finding the shuttle, considering the high concentration of GSH (5 to 20 mM) available in most mammalian cells [172]. Besides, Grxs are highly efficient and specific for glutathione-mixed disulfides.

However, the glutathionylation could also be rate limiting for Grx system, as we have to use high levels of GSH to push the pathway. It is not known whether this monothiol reaction is the major mode of R1 reduction by Grx, and whether this glutathionylation step is catalyzed by any other enzyme *in vivo*. There might be some unknown factors in the cell that could catalyze the glutathionylation; as an example, it has been reported that GSH S-transferase can induce glutathionylation [173]. It would be an advantage for tumor cells to have these factors or up-regulate them to use Grx system in parallel to Trx, since the Trx system is involved in several cellular pathways except acting as an electron donor for RNR. Moreover, there are number of tumor cells with almost no Trx expression [174].

A surprising variety of different functions has been discovered for Trx and Grx during last years apart from supplying electrons for ribonucleotide reduction. Cells have developed several systems to protect this essential metabolic pathway. For example in *E.coli*, a balanced supply of deoxyribonucleotides is obtained by a regulatory mechanism that up-regulates the level of RNR in response to the lack of any of its two main hydrogen donors, Trx1 or GSH plus Grx1 [175]. As described in details under 1.3.3 there are different potential electron donors for *E.coli* RNR [176, 177]; while in mammalian cells there are few known so far. It is not known whether the electron donor system is rate limiting for mammalian RNR activity. Probably the quite slow turnover we determined *in vitro* with the electron donors is a rate limiting step in dNTP synthesis.

So far a homologue of R2 called p53R2 is found in human/mouse cells and supposed to be involved in DNA repair and mitochondrial DNA synthesis [35]. It has to be established whether and how these electron donors function separately in DNA synthesis or repair in mammalian cells, and which of the two systems is dominant in different cell phases. To address these questions the kinetic analysis of both systems with R1-p53R2, as the active form of RNR for DNA repair and mitochondrial DNA

synthesis, should be performed to see possible differences in turnover number of Grx and Trx with the S-phase model (R1-R2). The co-localization of RNR and Trx/Grx in mammalian tissues also remains to be demonstrated [178, 179].

Overall RNR is a slow enzyme, routinely each subunit must be assayed in the presence of a large excess of the other one and it appears that the association of the two subunits in the active complex is weak. For synthesis of 3 billion base pairs during an S-phase in a mammalian cell, the rate of nucleotide production is calculated to be at least 140 nM s^{-1}. This is far away from the RNR activity that we measured with the Trx system as electron donor, and the k_{cat} calculated for the Trx system will only be sufficient for the synthesis of 30 percent of the dNTPs required for DNA replication.

The complexities outline above makes relating our *in vitro* results to *in vivo* very challenging. Is the activity of our recombinant enzymes comparable to the real activity of the enzyme, and are there any factors missing in the system? Grx system could be masked *in vitro* by the high activity of the Trx system, and the activity of the enzyme *in vivo* may have higher turnover with this system. The calculated k_{cat} for Grx is 1-2 per min which is barely good for S-phase, but if the enzyme has only 10 percent activity *in vitro*, Grx is also enough.

Intracellular form of the enzyme may consist of a tight complex of R1 and R2, possibly stabilized by other unknown intracellular factors. The highly active enzyme *in vivo* remains to be isolated and characterized as for *E.coli* [180]. The calf thymus enzyme was also slow and the calculated kinetics for Trx and Grx was lower than the recombinant enzyme [132, 133]. So far in *E.coli* the active form of the enzyme is obtained by working with highly concentrated protein solutions and by applying very gentle methods of bacterial lysis (10 to 20- fold higher activities) [180]. Probably as discussed for *E.coli* RNR, the stabilization of the more active enzyme needs some other factors, like membrane structures, or an additional protein or low molecular weight cofactor missing in our RNR assays.

The other feature of the R1 subunit is that the narrow cleft of the active site, excludes direct reduction of the active site disulfide by external redoxins [181]. A mobile shuttle designed in nature to be located in C-terminal of R1 [44], solves this in a way that, first the shuttle bends and exchanges the reducing equivalents from its cysteine residues to the active site. Consequently, the shuttle flanks away, and the

resulting C-terminal disulfide is reduced by an external thiol-dependent reductase system. This C-terminal region vary in length between species [44] and sequence alignment of the C-terminal of R1 from different species shows various number of amino acid residues between the cysteines; two intervening residues in human/mouse while *E.coli* has four intervening residues. It is assumed that different spacing between the two C-terminal cysteine residues in eukaryotic R1 and *E.coli* could dictate the difference in the contribution of Trx and Grx system in reduction of RNR. Further experiments using site-directed mutagenesis could be used to study this.

Much more needs to be done to determine whether and how this difference in C-terminal region could have an effect on enzyme activity. Understanding the mechanistic role of the shuttle will require making mutant human R1 by changing the spacing between the two C-terminal cysteine to resemble *E.coli* and see whether these modifications could alter the GSH-dependent mechanism of Grx, and other enzyme kinetics for mammalian enzyme.

Probably the crystallization of mammalian R1 not yet done, could answer part of these questions. RNR could be co-crystallized with GSH or Trx. Since the last residues of the C-terminal region of R1 are not visible in the X-ray crystallography of *E.coli* R1, consistent with the suggested flexibility of the shuttle, the synthesized peptide of this region could be evaluated. It is interesting to demonstrate the interaction of this part with human Trx/Grx, like what has been done for *E.coli* Grx [182]. The role of difference in spacing between the two C-terminal cysteine residues in *E.coli* and eukaryotic R1 could be investigated in this way.

Still several unclear issues remained to be clarified, such as: Is the R1 alone a substrate for Trx or it should be in complex with R2? Does R2 compete with Trx or Grx for binding to R1, and could this offer any explanation for different turnover numbers? Does the rereduction of the shuttle occur when the enzyme is in its complex form of both subunits, or on R1 before binding to R2? And is the shuttle disulfide interacts with the active site of the same R1 monomer, or does it interact with the other one? Molecular details of subunit interactions essential for answering these questions are not yet available but should be studied.

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APPENDIX (ARTICLES I-IV)