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Title: Regulation of Mouse Ribonucleotide Reductase by Allosteric Effector-Substrate Interplay and Hypoxia

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Abstract approved: _____

Christopher K. Mathews

In order to maintain genetic stability in eukaryotes, tight regulation of the relative sizes of deoxyribonucleoside triphosphate (dNTP) levels inside the cell is essential for optimal fidelity of DNA replication. Ribonucleotide reductase (RNR) is the enzyme responsible for proportional production of DNA precursors. Studies on regulation of this enzyme, the focus of this thesis, are important because mutations affecting RNR control mechanisms result in dNTP pool imbalance, thus promoting mutagenesis.

By using mouse RNR as a model for mammalian forms of the enzyme, three major factors—allosteric effectors, rNDP substrate concentrations, and hypoxic conditions—that influence the substrate specificity of RNR have been investigated. Allosteric regulation has been studied by the four-substrate assay,

which permits simultaneous monitoring of the four reactions catalyzed by this enzyme in one reaction mixture. Individual dNTPs affect the four activities differentially in a concentration-dependent manner with discrete effects of dTTP and dGTP on reduction of ADP and GDP, respectively. Ribonucleoside diphosphate (rNDP) substrate concentrations are equally important, as their variations lead to different product ratios. Results from nucleotide binding assays indicate that rNDPs directly influence binding of dNTP effectors at the specificity site, one of the two classes of allosteric sites, whereas ADP has an indirect effect, displacing other substrates at the catalytic site and consequently removing effects of those substrates upon dNTP binding. Hence, this is the first evidence of a two-way communication between the catalytic site and the specificity site. Oxygen limitation also plays an important role in controlling the enzyme specificity. Reactivation of the enzyme at different oxygen tensions, after treatment of the enzyme with hydroxyurea (HU) followed by removal of HU, reveals a distinct sensitivity of GDP reductase to low O_2 levels. Although the basis for specific inhibition of GDP reduction remains to be determined, some possibilities have been ruled out.

This research proves that in addition to allosteric regulation by nucleoside triphosphates, mouse RNR is also controlled by other factors. Since these components can simultaneously exert their effects upon enzyme specificity, complex regulatory patterns of RNR to provide a proportional supply of the DNA building blocks *in vivo* are suggested.

Regulation of Mouse Ribonucleotide Reductase by Allosteric Effector-Substrate Interplay and Hypoxia

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Korakod Chimploy

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REGULATION OF MOUSE RIBONUCLEOTIDE REDUCTASE BY ALLOSTERIC EFFECTOR-SUBSTRATE INTERPLAY AND HYPOXIA

Chapter 1

Introduction

During the processes of DNA replication and DNA repair, adequate and proportionally balanced deoxyribonucleotide levels are required. Unbalanced dNTP pools result in nucleotide misincorporation, lack of DNA replication fidelity, and thus enhanced mutation frequencies (Mathews and Ji, 1992). Without a sufficient supply of dNTPs, DNA repair can also be impaired. Therefore, production of dNTP *in vivo* needs to be tightly regulated.

Deoxyribonucleotides, the precursors for DNA synthesis, are produced by direct reduction of the corresponding ribonucleotides. The enzyme that is responsible for this process is ribonucleotide reductase (RNR), a single enzyme that simultaneously reduces all four substrates. Figure 1.1 shows dNTP synthetic pathways in which several enzymes are involved (Kunz *et al.*, 1994). Specifically, ribonucleotide reductase, the enzyme of interest in this dissertation, converts ribonucleoside diphosphate (rNDP) substrates to deoxyribonucleoside diphosphate (dNDP) products, which subsequently are phosphorylated by a single enzyme, nucleoside diphosphate kinase (NDPK) to the triphosphate species. Three of

the four dNTPs, 2′-deoxyadenosine 5′-triphosphate (dATP), 2′-deoxycytidine 5′-triphosphate (dCTP), and 2′-deoxyguanosine 5′-triphosphate (dGTP) are formed by this simple pathway.

The production of thymidine 5′-triphosphate (dTTP), in mammalian cells, is obtained by two different routes. One, referred to as the primary source of dTTP, is via deamination of 2′-deoxycytidine 5′-monophosphate (dCMP), resulting in 2′-deoxyuridine 5′-monophosphate (dUMP). Then the enzyme dTMP synthase (or thymidylate synthase, TS) carries out conversion of dUMP to thymidine 5′-monophosphate (dTMP). Along with this reaction, a tetrahydrofolate (THF) cofactor, 5,10-methylene-THF also becomes oxidized to dihydrofolate (DHF). To begin another cycle of dTMP synthesis, serine hydroxymethyltransferase catalyzes the conversion of THF to 5,10-methylene-THF. To produce dTTP from dTMP, two subsequent phosphorylation events by two distinct nucleotide kinases are involved. The other route by which dTTP is produced is via the RNR-catalyzed reduction of uridine 5′-diphosphate (UDP). 2′-deoxyuridine 5′-diphosphate (dUDP), formed by RNR, undergoes phosphorylation by NDPK, leading to formation of 2′-deoxyuridine 5′-triphosphate (dUTP) which, in turn, is metabolized by dUTPase to dUMP, the junction between the two pathways. The steps from there on are the same as in the first pathway, leading to the formation of dTTP.

Since RNR catalyzes the first reaction committed to DNA synthesis, and since this one enzyme participates in the synthesis of all four dNTPs, this enzyme

is thought to play a critical role in maintaining production of the four deoxyribonucleotides at rates proportional to the base composition of the genome (Jordan and Reichard, 1998). However, are the rates of synthesis and utilization always balanced *in vivo*? Interestingly, in mammalian cells, a natural asymmetry of the dNTP pool sizes is evident. In most cell lines analyzed, the dGTP pool size is always underrepresented (5-10% of total dNTPs). Moreover, the pool sizes are heavily biased toward dATP and dTTP, in general (Mathews and Ji, 1992; Traut, 1994).

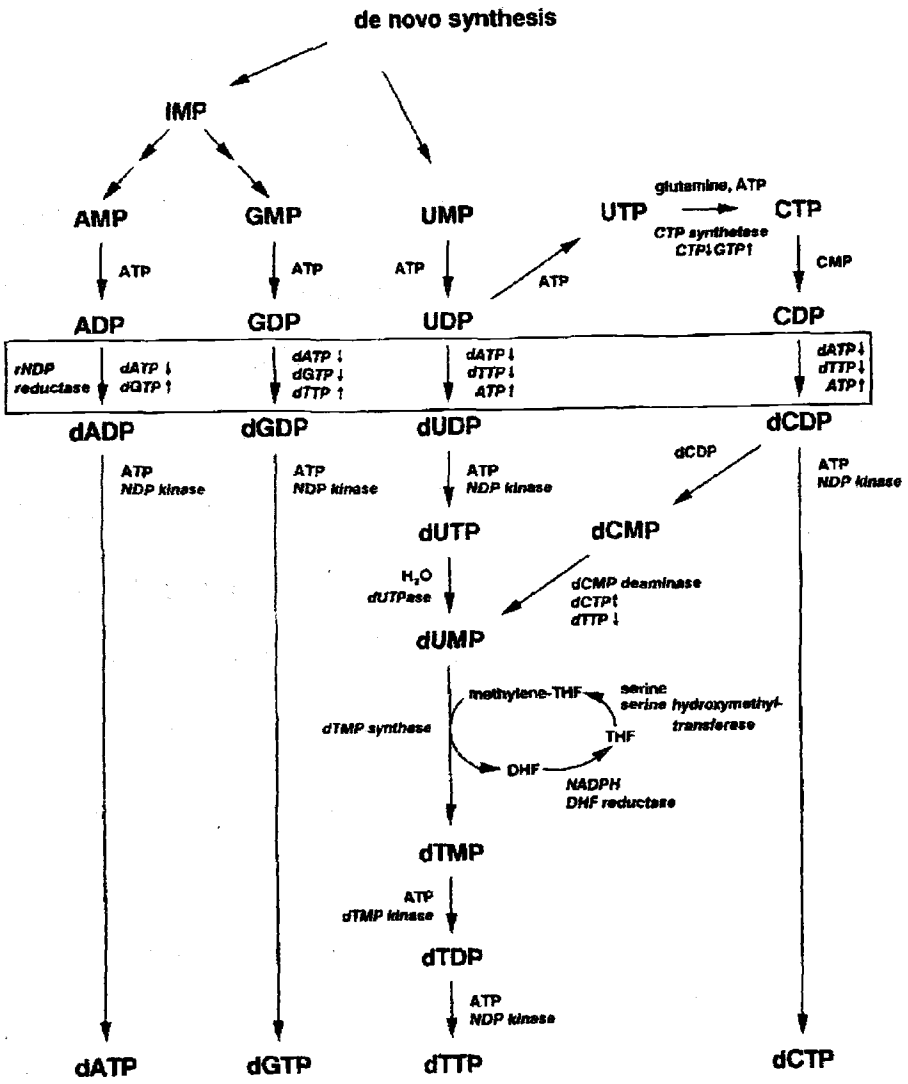


Figure 1.1 dNTP biosynthetic pathways in mammalian cells. This metabolic network starts with IMP and UMP, the products of the de novo purine and pyrimidine synthesis. Reactions catalyzed by ribonucleotide reductase (RNR) are regulated by nucleotide triphosphates shown in the Figure with adjacent upward and downward arrows as allosteric activators or inhibitors, respectively (Kunz *et al.*, 1994).

Although the basis for this natural imbalance of dNTPs has not yet been elucidated, perhaps, RNR or the pathways downstream contribute to this cellular phenomenon. Whatever the cause(s) might be, it is worthwhile to examine one pivotal possibility, regulation of mammalian RNR. Specifically, effects of three major factors, allosteric modifiers, rNDP substrates, and oxygen limitation upon the enzyme activity and substrate specificity have been investigated in this thesis.

Mammalian RNR, a member of Class I RNRs, is a heterodimer containing two homodimer subunits—R1 and R2. The small subunit, R2 contains a catalytically essential tyrosyl free radical and an oxygen-linked diferric iron center. Both are crucial for enzyme activity. The large subunit, R1, on the other hand, harbors the catalytic site to which rNDP substrates bind and two allosteric sites, the activity site and the specificity site to which nucleoside triphophates bind. Binding of ATP or dATP to the activity site affects the overall activity of the enzyme, whereas binding of ATP, dATP, dGTP and dTTP determines the substrate preference (Reichard, 1993).

Allosteric regulation of RNR, including the mammalian forms, by nucleoside triphosphates has been well documented (Eriksson *et al.*, 1979; Reichard *et al.*, 2000). However, those studies have been carried out by using an RNR assay mixture that contains one substrate at a time (a so called single-substrate assay). *In vivo*, the enzyme is exposed to all four substrates and allosteric modifiers simultaneously; hence, using a tool that allows simultaneous monitoring of all four reductase activities is more informative. Recently, the four-substrate

RNR assay has been developed in our laboratory and used to analyze bacteriophage T4 (Hendricks and Mathews, 1997) and vaccinia virus (Hendricks and Mathews, 1998) RNRs. In this thesis, this powerful tool has been used to investigate how mouse RNR is allosterically controlled by nucleoside triphophates under conditions that mimic physiological environments. In addition, effects of variations in dNTP concentrations upon the enzyme specificity have also been explored.

Another question addressed is whether rNDP substrate concentrations have any regulatory effects on the enzyme specificity. Suggestive evidence has arisen from the inhibitory effect of ADP on reduction of ADP itself seen with vaccinia virus RNR (Hendricks and Mathews, 1998). Here, it is of interest to determine whether this is the case for mammalian enzymes. The relative turnover rates of mouse RNR under various nucleotide environments have been measured. Besides, the competition among rNDP substrates as well as influence of substrates upon interactions with nucleotide effectors has been demonstrated.

Finally, effects of oxygen tension upon mouse RNR specificity have been investigated. Although Class I RNRs can function only under aerobic conditions, intracellular O_2 content sensed by the enzyme is much lower than atmospheric O_2. Therefore, it is interesting to learn how the enzyme responds to such limited O_2 levels. Besides, in many mammalian cell lines, consistent observations have been made regarding dCTP depletion at low O_2 (Probst *et al.*, 1988; Brischwein *et al.*, 1997; Chimploy *et al.*, 2000). It has been proposed that the specific effect of low O_2 upon dCTP production is basically due to a special sensitivity of CDP reduction

by RNR. In other words, RNR may also serve as a pO_2 sensor, transmitting the signal (i.e. high dCTP concentration) to the nuclear-replication machinery (Brischwein *et al.*, 1997). Thus, another objective of this research is to test this hypothesis.

Chapter 2

A Review of the Literature

General Characteristics of the Three Classes of Ribonucleotide Reductases

Based on differences in their structures and mechanisms used for radical generation, ribonucleotide reductases are divided into three major groups—Class I, II, and III (Reichard, 1993; Reichard, 1997). Class I enzymes are subdivided into two subgroups—Class Ia and Class Ib, according to their variations in the presence of redox cofactors and allosteric regulation (Jordan *et al.*, 1996).

As summarized in Table 2.1, Class I enzymes, found in most prokaryotes and higher organisms, are oxygen-dependent, thus can function only under aerobic conditions. They are composed of two nonidentical, homodimeric subunits called R1 and R2. The large subunit, R1 of Class Ia (α_2) is encoded by the *nrdA* gene, whereas the small subunit, R2 (β_2) is encoded by the *nrdB* gene (Fontecave *et al.*, 1992). The R1 protein is designated as the "business end" of the enzymes because it contains the catalytic site for substrate binding and the specificity site for binding of allosteric modifiers. Hence, this subunit is where catalysis and allosteric regulation take place. The R2 protein, on the other hand, contains an oxygen-bridged diiron center and a stable tyrosyl free radical that are essential for

enzyme activity (Reichard, 1993; Reichard, 1997). Its function is presumably to provide the tyrosyl radical utilized by the R1 for activation of the ribose ring of the rNDP substrates.

Like Class Ia, Class Ib enzymes are $\alpha_2\beta_2$ heterotetrameric. However, the genes that encode the large and small subunits are different: R1 (α_2) is encoded by *nrdE* and R2 (β_2) is encoded by *nrdF*. Another distinct feature of Class Ib involves regulation of overall activity. Interestingly, this subclass is not inhibited by dATP. It has been reported that CDP reduction by Class Ib *Salmonella typhimurium* RNR actually is activated by dATP at concentrations that completely inhibit the Class Ia enzymes. Furthermore, this particular enzyme harbors only two, instead of three, nucleotide binding sites—one catalytic site and a single specificity site. Since all known Class Ib enzymes appear to lack the last 50-60 amino acids at the N-terminus, it has been proposed that this region is where the activity site maybe located (Eliasson *et al.*, 1996). Taken together, alloteric control of Class Ib RNRs occurs through the specificity site only.

Class II RNRs, found only in microorganisms, are functional under both aerobic and anaerobic environments. The first Class II RNR was discovered in *Lactobacillus leichmannii* in 1964 (Blakley and Barker, 1964). Important characteristics that make this class of enzymes unique include their structures, adenosylcobalamin-dependence, and ability to use ribonucleoside triphosphates (rNTPs) as substrates. Unlike other classes, Class II functions as a α monomer or a homodimer, α_2, converting rNTPs to the corresponding dNTPs. Additionally,

like Class Ib enzymes, Class II is not inhibited by dATP, suggesting that the second allosteric binding site, the overall activity site is also absent (Chen *et al.*, 1977). The fact that this class of enzymes lacks the small subunit does not limit its function. The radical generated by adenosylcobalamin, whose function is similar to the function of R2, facilitates reduction of rNTP substrates (Hamilton *et al.*, 1972). This is supported by the discovery of a transient cysteinyl radical formed in *L. leichmanni* RNR (Licht *et al.*, 1996).

Class III RNRs are found in microorganisms that can grow anaerobically, including *E. coli* (Fontecave *et al.*, 1989) and bacteriophage T4 (Young *et al.*, 1994). The genes that encode the large and small subunits are *nrdD* and *nrdG*, respectively. This class of enzymes is structurally similar to Class I enzymes ($\alpha_2\beta_2$); however, it contains an iron-sulfur cluster, instead of the oxygen-linked diiron center in the R2 subunit. This iron-sulfur cluster, together with S-adenosylmethionine, generates an oxygen-sensitive glycyl radical, essential for enzyme activation in the R1 subunit (Ollagnier *et al.*, 1996). Like Class II enzymes, the Class III RNRs require rNTPs as substrates. Interestingly, they use formate as the source of reducing equivalents (Mulliez *et al.*, 1995). Dithiothreitol (DTT), a nonphysiological reductant (Ollagnier *et al.*, 1999) or thioredoxin, the physiological counterpart (Padovani *et al.*, 2001) have also been shown recently to serve as reducing agents during activation of the Class III enzymes.

Table 2.1 A summary of differences between the three classes of ribonucleotide reductases (Jordan and Reichard, 1998; Ollagnier *et al.*, 1999; Padovani *et al.*, 2001).

	Class Ia	Class Ib	Class II	Class III
Oxygen dependence	Aerobic	Aerobic	Aerobic/ Anaerobic	Anaerobic
Structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha(\alpha_2)$	$\alpha_2\beta_2$
Genes	*nrdAB*	*nrdEF*	*nrdJ*[a]	*nrdDG*
Radical	Tyr...Cys	Tyr...Cys	AdB12...Cys	AdoMet ...Gly...Cys
Metal site	Fe-O-Fe	Fe-O-Fe Mn-O-Mn	Co	Fe-S
Substrate	NDP	NDP	NDP/NTP	NTP
Reductant	Thioredoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	Thioredoxin	Formate, DTT Thioredoxin
Allosteric sites/ polypeptide chain	2	1	1	2
dATP inhibition	Yes	No	No	Yes
Occurrence	Eukaryotes Eubacteria Bacteriophages Viruses	Eubacteria	Archaebacteria Eubacteria Bacteriophages	Archaebacteria Eubacteria Bacteriophages
Prototype	*E. coli* Mouse	*S. typhimurium* *C.ammoniagens*	*L. leishmannii*	*E. coli*

[a]The gene encoding ribonucleotide reductase from *T. maritima* is named *nrdJ* (Jordan *et al.*, 1998).

In an evolutionary aspect, it has been proposed that the three classes of RNRs have a common ancestral origin for the large subunit, R1 due to their similarities in reaction mechanisms and allosteric regulation (Reichard, 1993). In fact, the Class III enzymes have been suggested as the common ancestor of the modern RNRs for several reasons. First, this class of enzyme can function only under anaerobic conditions, which means that it could have been the first class of enzymes operative during evolution before the atmosphere has been filled with oxygen. Second, the glycyl radical is generated by an iron-sulfur cluster, and a combination of iron and sulfur may have evolved very early during the primordial environment (Wächtershäuser, 1990). Third, the external reducing equivalent utilized by Class III RNRs is formate, a simple organic reductant speculated to be available on primitive earth. As oxygen levels increase in the atmosphere, Class III enzymes are no longer functional. Therefore, Class II RNRs, which can function under both anaerobic and aerobic conditions, have arisen, followed by the Class I enzyme, whose function is oxygen-dependent.

Allosteric Regulation of Three Classes of Ribonucleotide Reductases

Among the three classes of RNRs, regulatory patterns by nucleoside triphosphates are highly conserved (Figure 2.1). The large subunit of Class Ia RNRs contains two allosteric sites—the activity site and the specificity site (Brown and Reichard, 1969). Binding of ATP or dATP at the activity site (also called

the low affinity site) controls the overall activity of the enzyme. That is, binding of ATP at the activity site activates the enzyme, while binding of dATP at the same site is inhibitory. However, binding of ATP or dNTPs at the specificity site (also called the high affinity site) determines the substrate preference of the enzyme. For example, dATP (at low concentrations) activates reduction of CDP and UDP, dGTP activates reduction of ADP, and dTTP activates reduction of GDP.

Binding of allosteic modifiers at the specificity site of Class Ib and Class II RNRs results in the same allosteric behavior as the Class I enzymes. However, since Class Ib and Class II enzymes harbor only the specificity site, they are not inhibited by dATP (Eliasson *et al.*, 1996). By contrast, allosteric control of Class III RNRs is very similar to that of Class I, although Class III enzymes require rNTPs as substrates like Class II. Class III RNRs contain two allosteric sites that affect both overall activity and substrate specificity, and hence, cannot be identified as either an activity site or a specificity site. As a result, one is called the pyrimidine site, and the other the purine site. It has been reported that binding of ATP at the pyrimidine site stimulates reduction of CTP and UTP. However, binding at the purine site of dGTP and dTTP activates reduction of ATP and GTP, respectively (Eliasson *et al.*, 1994).

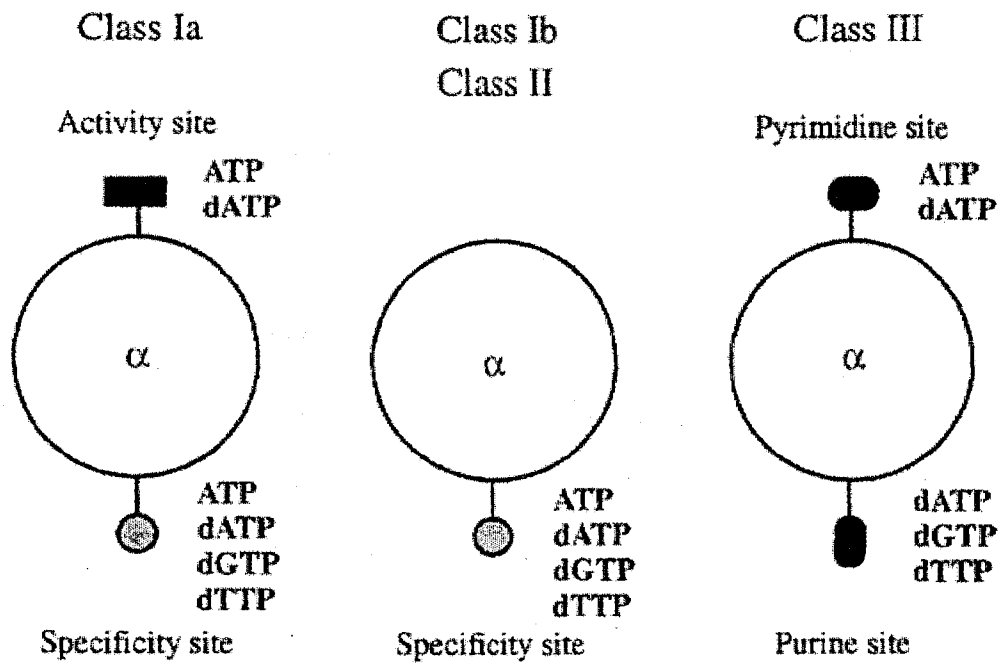


Figure 2.1 Models for the allosteric regulation of the three classes of ribonucleotide reductases proposed by Eliasson and colleagues (1996). Nucleotide binding sites are shown per monomer of the enzyme.

Biochemistry of Ribonucleotide Reductases

Catalytic Mechanism of Ribonucleotide Reduction

All three classes of RNRs catalyze the reduction of ribonucleotides to the corresponding deoxyribonucleotides by using a unique radical mechanism. For Class I and Class II enzymes, small proteins with redox-active thiols—thioredoxins and glutaredoxins—serve as external electron donors (Holmgren and Björnstedt, 1995; Holmgren and Åslund, 1995). *In vitro*, the electrons can also be provided by dithiothreitol (DTT) but with lower efficiency (Berglund, 1972) since it bypasses a redox-active cysteine pair, and directly reduces the cysteines at the active site after each cycle of catalysis (Mao *et al.*, 1992). The ultimate reducing equivalent, however, comes from NADPH, which reduces the cysteines of thioredoxins and glutaredoxins via a specific enzyme, thioredoxin reductase or glutathione plus glutathione reductase, respectively. Class Ib enzymes utilize a protein called the NrdH-redoxin as a specific reductant (Jordan *et al.*, 1996; Jordan *et al.*, 1997), whereas Class III RNRs use formate, instead of a redoxin (Mulliez *et al.*, 1995).

As illustrated in Figure 2.2, the reaction pathway is started by substrate binding at the catalytic site of the enzyme in a reduced form. In *E. coli* RNR, this binding of the substrate leads to an oxidized cysteinyl radical formation as a result of a transfer of a radical function from Y122 of the small subunit (R2) to C439 of

the large subunit (R1). The transient radical then initiates the reduction of the ribonucleotide by abstracting the hydrogen from the 3' position of the ribose ring, generating a substrate radical. Protonation of the 2'-hydroxyl group of the ribose then occurs, followed by loss of H_2O from this position. As a result, a substrate cation radical is formed. This substrate cation is reduced, consequently, by the redox-active cysteine pair C225 and C462. Finally, to complete the reaction, the originally abstracted hydrogen (at C439) is returned to the 3' position of the substrate with regeneration of the thiol radical at C439 (Stubbe, 1990).

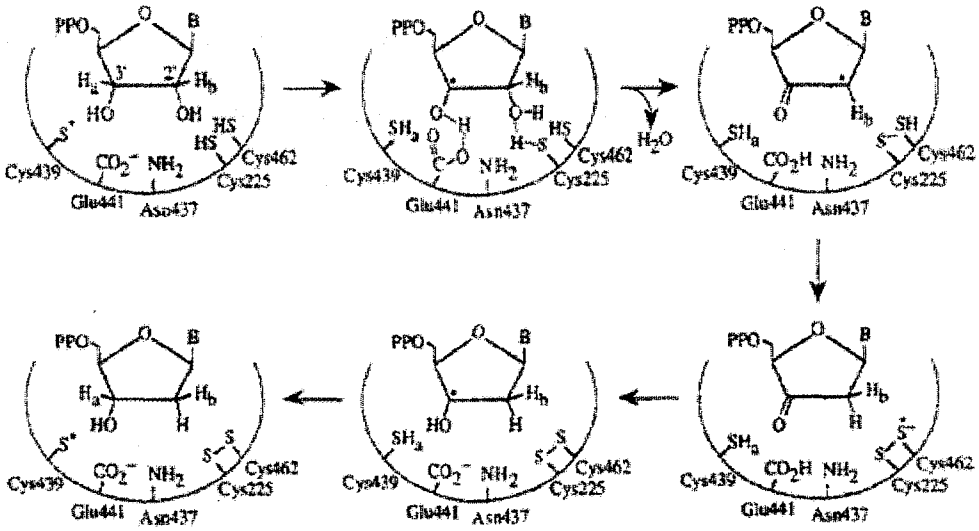


Figure 2.2 The catalytic mechanism of ribonucleotide reductase (Stubbe, 1990; Mao *et al.*, 1992).

Formation of the Thiyl Radical

In Class I RNRs, the thiyl radical is transiently formed via a long-range electron transfer from Y122 of the R2 protein to C439 of the R1. Participation of other residues on both subunits include the iron center of the R2 (D237, W48, and Y356) and the tyrosine residues Y731 and Y730 of the R1 (Uhlin and Eklund, 1996; Eriksson *et al.*, 1997; Ekberg *et al.*, 1996; Persson *et al.*, 1996).

The thiyl radical in Class II enzymes is generated from adenosylcobalamin during catalysis. In *L. leishmannii*, for instance, the thiyl radical is formed by a concerted pathway, resulting in formation of 5'-deoxyadenosine and cob(II)alamin, with the thiyl radical very close to cob(II)alamin (Licht *et al.*, 1996).

In Class III enzymes, more evidence on how the thiyl radical is formed is needed. However, it is suggested that a transient thiyl radical is involved, and that the stable glycyl radical stores the unpaired electron and does not catalyze the abstraction of a hydrogen from the 3' position (Eliasson *et al.*, 1995).

Formation of the Stable Radicals

For Class I enzymes, generation of the tyrosyl radical is linked to the diiron center, starting from either apoR2 (iron-free) or metR2 (with an intact diiron center). To apoR2, only ferrous iron and oxygen need to be added, while in metR2,

the diiron center needs to be reduced before the radical can be formed by oxygen (Nordlund *et al.*, 1990; Nordlund and Eklund, 1993). In *E.coli*, it is suggested that a flavin reductase reduces riboflavin, FMN or FAD. The ferric iron of metR2 is, in turn, reduced by these reduced flavins. The tyrosyl radical can then be generated by oxygen (Fontecave *et al.*, 1989; Coves *et al.*, 1995).

Generation of the glycyl radical in the R1 protein of Class III reductases is rather interesting since the Class II enzymes contain the iron-sulfur cluster, instead of the oxygen-linked diiron center. The reaction starts in the R2 subunit with reduction of the iron-sulfur cluster enzymatically by the flavodoxin reducing system or chemically by dithionite or deazaflavin and light (Ollagnnier *et al.*, 1997). The reduced 4F-4S cluster then facilitates a tight binding of S-adenosylmethionine to the small subunit, forming the glycyl radical on the large subunit.

Stoichiometry for the Formation of the Tyrosyl Radical and Oxygen-Bridged Dinuclear iron Center

The role of oxygen in forming the catalytically essential tyrosyl radical and the diferric iron center is well studied both in the mouse (Ochiai *et al.*, 1990) and the *E. coli* (Bollinger *et al.*, 1991) R2 proteins of the Class I enzymes. The stoichiometry of the tyrosyl radical and iron center assembly can be summarized as follows.

$$Tyr + 2\ Fe^{II} + O_2 + e^- + 2\ H^+ \rightarrow Tyr\bullet + Fe^{III}\text{-}O\text{-}Fe^{III} + H_2O$$

In this reaction, two electrons are derived from the oxidation of two ferrous irons to two ferric irons. The tyrosyl radical is formed by oxidation of the tyrosine residue on the R2 protein, giving out another electron. Production of the fourth electron depends on reductants present. For example, without any other reducing agents, the fourth electron comes from ferrous iron, indicating the ratio of oxidized iron to tyrosyl radical theoretically being 3 to 1. Ascorbate can function as an electron donor, giving out the fourth electron when added to the reaction mixture. In this case, the ratio of oxidized iron to tyrosyl radical becomes 2 to 1. Experimentally, it has been demonstrated that formation of one mole of tyrosyl radical in the mouse protein requires 3.5 ± 0.5 moles of ferrous iron, in the absence of other reductants. However, in the presence of ascorbate, 2.6 ± 0.4 moles are required. Similar results have been shown for the *E. coli* protein. That is, 3.2 ± 0.1 and 2.5 ± 0.1 moles of ferrous iron are required in the absence and presence of ascorbate, respectively. These data suggest that the assembly of the tyrosyl radical and diferric iron center in mouse R2 is similar to that in *E. coli* protein.

Mammalian Ribonucleotide Reductases

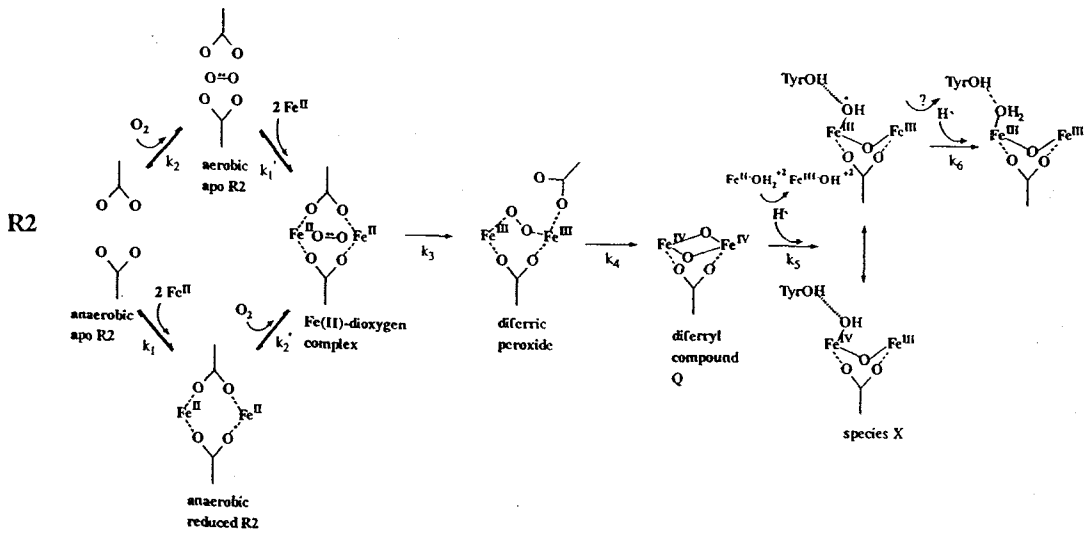
Like RNRs in other organisms with Class I enzymes, the mammalian RNR consists of two nonidentical homodimeric subunits, R1 and R2. Typically, the large

subunit, R1 (two 90-kDa polypeptides) contains the binding sites for nucleoside triphosphate allosteric effectors and rNDP substrates, whereas the small subunit, R2 (two 45-kDa polypeptides) harbors a dinuclear iron center and in its active form, a tyrosyl free radical (Thelander *et al.*, 1980). The functional gene encoding the mouse R1 protein is mapped to chromosome 7, and full-length mouse cDNA has been cloned and sequenced (Caras *et al.*, 1985; Thelander and Berg, 1986; Brissenden *et al.*, 1988). The mouse R2 gene, on the other hand, is mapped to chromosome 12, and also has been cloned and sequenced (Yang-Feng *et al.*, 1987; Thelander and Thelander, 1989).

A Radical Transfer Pathway in Mouse Small Subunit

The free radical has been characterized in mouse fibroblast cell extracts to be localized to a tyrosine residue (Gräslund *et al.*, 1982). It has been reported that the mR2-specific electron paramagnetic resonance (EPR) signal resembles the signal of the bacteriophage T4 radical. However, unlike the radicals of T4 or *E. coli*, the mouse R2 radical can be regenerated by the addition of dithiothreitol (DTT) in the presence of oxygen to the cell extract whose radical previously was destroyed by hydroxyurea. By site-directed mutagenesis, the tryptophan and aspartic residues on the mouse R2 have been identified as essential links between the active site on R1 and the tyrosyl radical on R2 in a long-range electron transfer (Rova *et al.*, 1995). The W103Y and D266A mutant proteins can still form a

normal tyrosyl radical, whereas the W103F protein forms only low amounts of radical. Besides, the mutations do not cause any changes in either the kinetic rate constants or the equilibrium dissociation constant of the R1/R2 complex. However, all mutant proteins become enzymatically inactive, supporting the importance of these amino acid residues in an electron transfer process. Kinetic studies on the active mouse R2 reconstitution have proven that the long-range electron transfer pathway is involved in the tyrosyl radical formation (Schmidt *et al.*, 1998), which therefore has been called the radical transfer pathway. A proposed reaction mechanism is depicted in Figures 2.3 and 2.4. To understand the radical transfer pathway in more detail, the conserved tyrosine 370, located near the flexible C-terminus of the mouse R2 protein, has been altered by site-directed mutagenesis. The Y370F mutant protein leads to inactivation of the R1/R2 complex, whereas the Y370W slows down the radical transfer process. These results indicate that Tyr^{370} is the connecting link in the intersubunit radical transfer pathway (Rova *et al.*, 1999).



Mouse R2: k_5 (or $k_{4 \text{ or } 3 \text{ or } 2 \text{ or } 1'}$) = 54 s^{-1}; k_6 = 45 s^{-1} at 25°C

Figure 2.3 The proposed mechanism for the formation of the diiron center without formation of Tyr•, a side reaction of the reconstitution *in vitro* (Schmidt *et al.*, 1998).

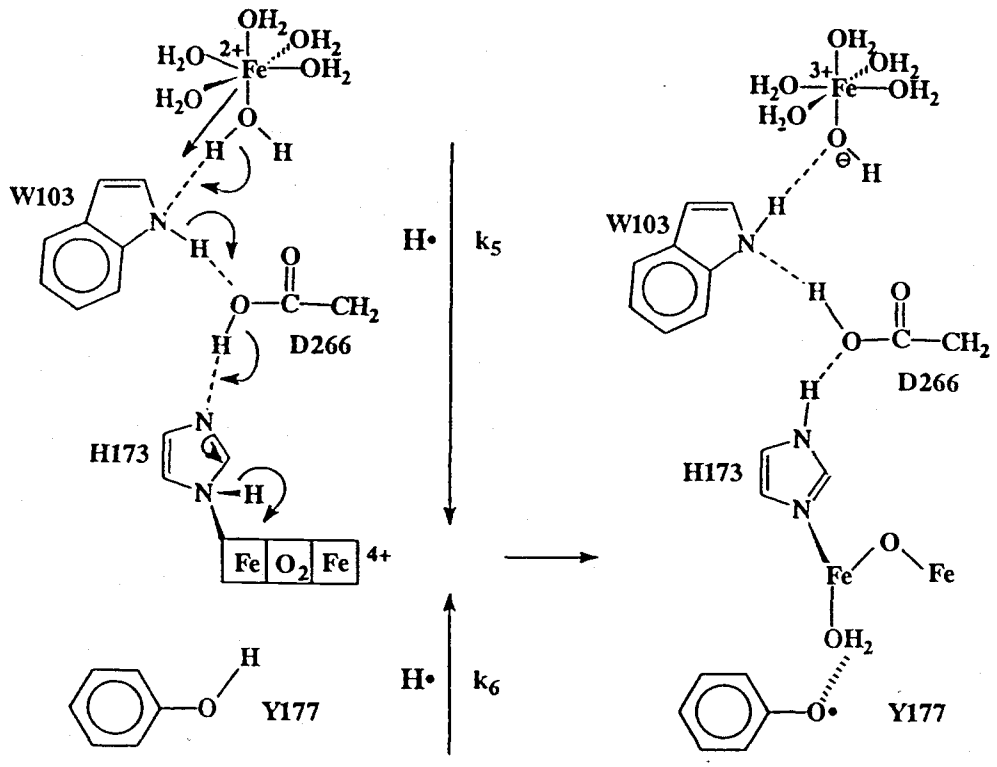


Figure 2.4 The proposed model for the last step of the reconstitution of the mouse R2 (Schmidt *et al.*, 1998).

Mouse R1/R2 Complex Formation

Recombinant mouse R1 (Davis *et al.*, 1994) and R2 (Mann *et al.*, 1991) proteins have already been purified and characterized. Characterization of the purified mouse R2 indicates that like the R2 of herpes simplex virus, the mouse R2 protein contains approximately 50% α-helical structure. Moreover, the iron center/radical interactions in the mouse R2 are stronger than those in the *E.coli* protein.

Interactions between the R1 and R2 protein are crucial for enzyme activity. It has been demonstrated that the C-terminus of the mouse R2 protein is highly mobile in solution. However, as it is combined with R1 protein, this region becomes more rigid, suggesting that the mobility decrease is due to the binding of the R2 C-terminus to the R1 protein (Lycksell *et al.*, 1994). Later on, it was shown that the region on the R1 involved in the interactions with R2 is the C-terminus (Davis *et al.*, 1994). What facilitates the R1/R2 interactions? Studies on kinetics of R1/R2 complex formation reveal that allosteric modifiers significantly affect the R1/R2 interactions (Ingemarson and Thelander, 1996). All four nucleotides examined—ATP, dATP, dGTP and dTTP—apparently increase the affinity of the binding between R1 and R2. The affinity between the two subunits is much higher in the presence of ATP and dATP, presumably because these two nucleotides can bind at both activity site and specificity site. Since the interactions are the same at high concentrations of R1 protein in the absence of allosteric effectors as at low R1

concentration in the presence of modifiers, it is speculated that allosteric effectors influence R1/R2 complex formation by facilitating dimerization of the R1 monomers. Therefore, once the R1 dimer is formed, it binds more tightly to the R2 dimer. In these experiments, the molar stoichiometry between the mouse R1 and R2 proteins is close to 1:1.

Regulation of RNR Expression by the Cell Cycle

It has been reported that the activity of the holoenzyme is controlled by an S-phase specific *de novo* synthesis and subsequent breakdown of the R2 protein (Eriksson *et al.*, 1984; Engström *et al.*, 1985). Expression of mRNA levels of both R1 and R2 is undetectable in G_0/G_1 phase, but rises dramatically in parallel just before entering the S phase. The R1 and R2 mRNA levels then reach the same levels before declining as cells enter the G_2/M phases (Björklund *et al.*, 1990). Expression of the R1 protein, by contrast, is shown to be detectable only in proliferating cells, but the levels are constant throughout the cell cycle. This is possibly due to its long half-life (> 24 hours) (Mann, *et al.*, 1988) compared to a half-life of 3 hours for the R2 protein (Eriksson *et al.*, 1984).

dNTP Pool Imbalances and Ribonucleotide Reductase Mutations

In order to maintain high fidelity of DNA replication in mammalian cells, proper control of intracellular deoxyribonucleotide concentrations is crucial. It has been demonstrated that perturbations of balanced dNTP pool sizes can lead eventually to mutagenesis (Kunz *et al.*, 1994; de Serres, 1985). Besides, several factors, including genetic alterations of enzymes involved in *de novo* dNTP biosynthetic pathways, directly contribute to dNTP pool asymmetries (Meuth, 1989). As supporting evidence, three important mutants were isolated by using the cultured mouse T-lymphoma (S49) cell line (Weinberg *et al.*, 1981). Two of the mutant cell lines contain mutations affecting the large subunit (R1) of ribonucleotide reductase, whereas the other is deficient in dCMP deaminase. These mutations result in deranged dNTP levels and enhanced rates of spontaneous mutation compared to the control cells. Moreover, normalization of the dNTP pools of the mutants can suppress the mutator phenotype, suggesting that the mutation rates are directly influenced by the unbalanced dNTP pool sizes.

Further studies on the two RNR-defective mutants reveal some interesting characteristics. Both cell lines harbor altered allosteric properties; dGuo-200-1 is resistant to deoxyadenosine (Eriksson *et al.*, 1981a), whereas dGuo-L is resistant to deoxyguanosine (Ullman, *et al.*, 1981). What the two clones have in common is the fact that enzyme activities of dGuo-200-1 and dGuo-L are inhibited only 50% by dATP and dGTP, respectively, and both mutants were proven to possess two

protein R1 components—a mutant allele product and a wild type allele product—in approximately equal amounts. Furthermore, in comparing the allosteric properties of these mutants to those of wild type cells, there is genetic evidence that the two allosteric sites, the activity site and the substrate specificity site, are two independent allosteric regulatory domains (Eriksson *et al.*, 1981b). The discovery of these clones with genetically altered ribonucleotide reductase provides an intriguing basis for the study of allosteric regulation of such an important enzyme, which is the focus of this thesis.

Allosteric Regulation of Mammalian Ribonucleotide Reductases

Reduction of the four ribonucleoside diphosphates (rNDPs) to the corresponding deoxyribonucleoside diphosphates (dNDPs) is catalyzed by a single enzyme, ribonucleotide reductase. Binding of nucleoside triphosphate allosteric modifiers plays a crucial role in regulating the overall activity and the substrate specificity of the enzyme (Thelander and Reichard, 1979). Allosteric control of a purified mammalian RNR was first studied from calf thymus by using a single-substrate RNR assay (Eriksson *et al.*, 1979). In this experiment, it was found that ATP, a general activator is required for reduction of any substrates. Whereas dATP acts as a primary inhibitor, dTTP and dGTP are positive effectors for reduction of GDP and ADP, respectively. Furthermore, reduction of CDP and UDP are stimulated only by ATP.

By using the same method (a single-substrate assay) together with the nucleotide binding assay, allosteric regulation has been studied with mouse RNR (Reichard *et al.*, 2000). The results are in agreement with allosteric behaviors of *E. coli* and calf thymus RNRs. Studies with a mutated mouse R1 protein containing D57N show that dATP no longer inhibits CDP reduction. Instead, reduction of CDP, ADP and GDP are all stimulated by dATP. In addition, the two allosteric sites of the mutated R1 protein have the same low affinity for dATP, similar to that of the activity site of the wild-type. Because the mutated enzyme is activated by both ATP and dATP, it is suggested that a single mutation makes the enzyme unable to distinguish between the two effectors. Taken together, mutation in the active site leads to a decrease in the binding affinity for dATP, indicating cross-talk between the activity site and the specificity site (Reichard *et al.*, 2000).

Recent quantitative studies on allosteric control of mouse RNR help to achieve better understanding of complex regulatory patterns of mammalian RNRs. By using physical methods such as nucleotide binding assays, dynamic light scattering, analytical ultracentrifugation, sedimentation velocity and sedimentation equilibrium, quantitative models for allosteric regulation of mouse RNR have been proposed. The first model is called the 16 species model, in which 16 parameters have been determined (Scott *et al.*, 2001). According to this model, to understand how RNR is controlled, several factors must be taken into account. Those factors shown in this experiment are the effects of *i*) rNDP substrates *ii*) allosteric modifiers and *iii*) dimerization of the R2 subunit on the quaternary structure of the

R1 subunit and the dependence of holoenzyme ($R1_2R2_2$) activity on substrate and effector concentrations. Another model from the same laboratory emphasizes an oligomerization of the large subunit facilitated mainly by ATP and dATP (Kashlan *et al.*, 2002). Figure 2.5 below demonstrates the modulation of RNR activity by binding of ATP or dATP on the large subunit. Important features of this schematic diagram include: 1) Binding of ATP or dATP to the specificity (s-) site and the adenine-specific (a-) site causes formation of $R1_2$ and $R1_4$, respectively. 2) Two conformations of $R1_4$ are possible ($R1_{4a}$ and $R1_{4b}$) with $R1_{4b}$ predominating at equilibrium. 3) Binding of ATP at a novel site called hexamerization (h-) site results in formation of $R1_6$. 4) Both $R1_2/R2_2$ and $R1_6/R2_2$ complexes are enzymatically active, while a complex of $R1_{4b}/R2_2$ has little activity. Figure 2.6 illustrates the significance of the a-site in oligomerization of the R1 protein. The projections correspond to the N-terminal region of each R1 subunit responsible for the dimer-dimer contact. The model shows that formation of the R1 dimer results in an "open" form of $R1_4$ with only two or four dimer-dimer contacts. This open form then can either isomerize to a "close" form (with four contacts) or bind to the third $R1_2$, forming a "close" $R1_6$ with six dimer-dimer contacts. Although changes in R1 structure are modulated primarily by the two nucleoside triphosphates, addition of other nucleotides, including ADP, GTP, UTP, dTTP, dGTP and ddATP also influence the oligomerization of R1, which, in turn affects the enzyme activity.

Hence, a combination of effects of these factors, besides nucleotide triphosphate modifiers would certainly intensify the complexity of regulation of mammalian RNRs *in vivo*.

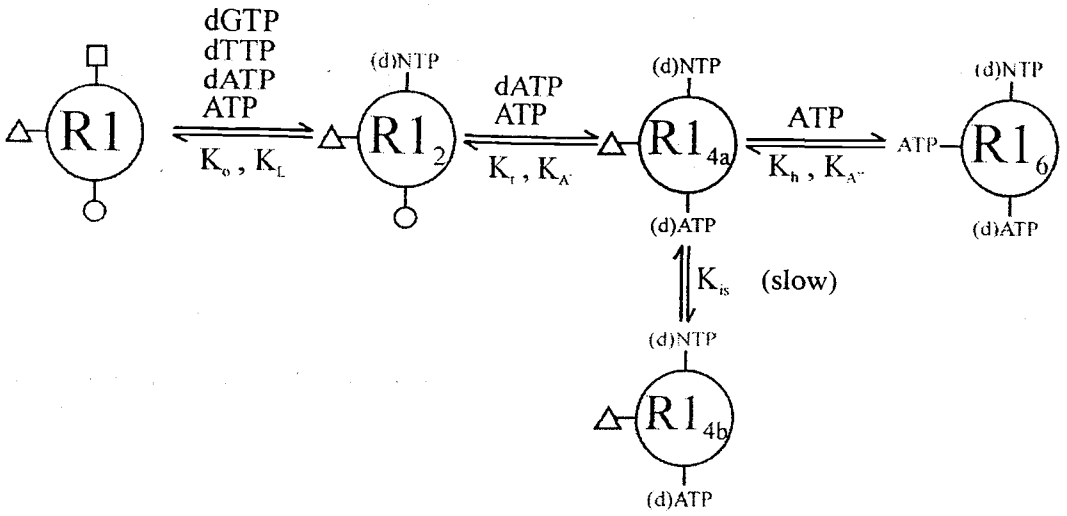


Figure 2.5 Effects of ATP and dATP upon the oligomerization of mouse ribonucleotide reductase (Kashlan *et al.*, 2002).

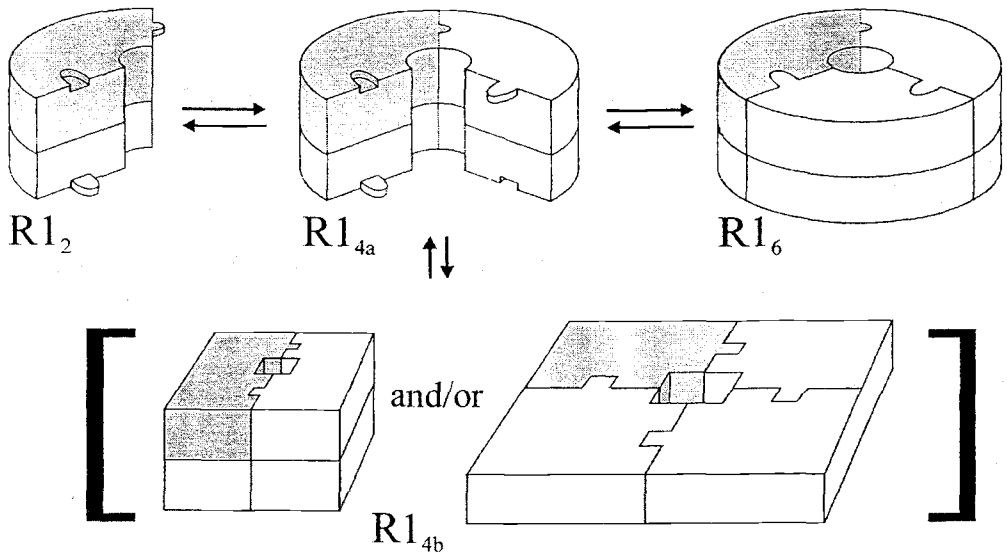


Figure 2.6 Significance of the a-site in the oligomerization of mouse ribonucleotide reductase (Kashlan *et al.*, 2002).

Chapter 3

Materials and Methods

Overexpression and Purification of Recombinant Ribonucleotide Reductase (RNR) Subunits

Overexpression of Mouse and Vaccinia Virus RNR Large Subunits

Escherichia coli (*E. coli*) BL21(DE3) pLysS carrying pETR1 (mouse RNR large subunit, mR1) was kindly provided by Dr. Lars Thelander (Umeå University, Sweden). The previously described expression system was used (Davis *et al.*, 1994). A single colony was inoculated as an overnight culture in Luria-Bertani (LB) medium containing 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol, and incubated at 30°C with aeration. The overnight culture was then diluted 1:100 in 1 liter of terrific broth (TB) medium, containing 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol, incubated at 30°C with aeration until optical density at 595 nanometer (OD_{595}) = 0.4-0.6. After adding 100 μl of 10% antifoam B (silicone emulsion), isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 100 μM (with 2 ml taken to serve as uninduced control). Another 100 μl of 10% antifoam B was added after an hour of induction at 15-18°C.

Induction was then continued for a total of 18 hours. Cells were harvested by centrifugation at 2,500 ×g, 4°C for 20 minutes.

Vaccinia virus RNR large subunit (vvR1) was overexpressed using essentially the same procedure except that an overnight culture of *E. coli* BL21(DE3) harboring pET11c.vvR1 was inoculated into 1 liter of LB (1:100 dilution) containing 100 μg/ml ampicillin, and incubated at 30°C with aeration until OD_{600} = 0.2-0.3 before induction. Hydroxyurea was also included at a final concentration of 5 mM during the induction to increase production of soluble vvR1 (Slabaugh *et al.*, 1993).

Overexpression of Mouse RNR Small Subunit

Mouse R2 protein was overexpressed as previously reported (Mann *et al.*, 1991). An overnight culture of *E. coli* BL21(DE3) containing pET3a.mR2 was inoculated 1:100 into 1 liter of LB in the presence of 50-100 μg/ml carbenicillin and 25 μg/ml chloramphenicol. After incubation at 37°C with aeration until OD_{590} = 0.4-0.6, cells were induced with 0.5 mM IPTG final concentration at 37°C for 4 hours. Cells were then harvested by centrifugation at 2,500 ×g, 4°C for 20 minutes.

Purification of Mouse and Vaccinia Virus R1 Proteins

To purify mouse R1 protein (Davis *et al.*, 1994), harvested cells were resuspended in 3 volumes of 50 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.3 containing 1 mM dithiothreitol (DTT) [dATP-Sepharose column buffer], lysed in a French pressure cell at 15,000 p.s.i., and centrifuged at 27,000 ×g, 4°C, for 30 minutes. Supernatant was saved. Fifty percent saturated ammonium sulfate ($(NH_4)_2SO_4$) precipitation (0.295 g/ml) was carried out followed by stirring on ice for 30 minutes and centrifuging at 12,000 ×g, 4°C for 10 minutes. The pellet was then dissolved in dATP-Sepharose column buffer, filtered through a 5-μm Versapor® Membrane (sterile Acrodisc® syringe filter; Pall Gelman Laboratory), and loaded onto a dATP-Sepharose column at a flow rate of 10 ml/hr. In sequence, the column was washed at a flow rate of 20 ml/hr with column buffer containing 500 mM potassium chloride (KCl) and 5 mM ATP. Mouse R1 protein was eluted by column buffer containing 75 mM ATP. The column was finally washed with column buffer containing 5 mM dATP. The protein was concentrated by using Centricon-30 or Centriprep-30 centrifugal filter devices (Amicon® Bioseparations) concentrating units, and tested for enzyme activity by measuring the reduction of ^{3}H-CDP (^{3}H-CDP enzymatic assay).

Vaccinia virus R1 was purified by using a similar procedure to mouse R1 purification. However, column buffer was composed of 50 mM HEPES, pH 8.2, 100 mM KCl, and 1 mM DTT. The $(NH_4)_2SO_4$ precipitation step was also omitted;

thus, filtered supernatant was directly applied to a dATP-Sepharose column. Similarly, after sequentially washing the column with column buffer containing 500 mM KCl and 5 mM ATP, the protein was eluted by 75 mM ATP and 5 mM dATP. Purified protein was concentrated and checked for enzyme activity as mentioned above.

Purification of Mouse R2 Protein

The purification protocol was modified from Mann *et al* (1991) and Howell *et al* (1992). Harvested cells were resuspended in 3-5 volumes of 50 mM HEPES, pH 7.6, 5% glycerol, and 2 mM magnesium chloride ($MgCl_2$), 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 2 mM DTT, and lysed in a French pressure cell at 15,000 p.s.i. After centrifugation at 10,000 ×g, 4°C for 20 minutes, 10% (w/v) streptomycin sulfate, pH 7.0, was added to the supernatant (while being gently stirred on ice) to a final concentration of 2.5%. Gentle stirring was continued (on ice) for 15 more minutes before centrifugation at 27,000 ×g, 4°C was carried out for 20 minutes. Ammonium sulfate precipitation (40% saturation) was then performed on supernatant followed by centrifugation at 27,000 ×g, 4°C for 30 minutes. The pellet was saved, resuspended in 3 ml of 50 mM Tris-HCl, pH 7.6, 1 mM PMSF, 1 mM (ethylene dinitrilo) tetra acetic acid (EDTA), and argon-purged gently before regeneration of a tyrosyl free radical and diiron center. A tyrosyl free

radical and diiron center were regenerated by adding 3 ml of 4 mM previously argon-purged ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) in 50 mM sodium ascorbate/50 mM Tris-HCl pH 7.6. After a 5-minute incubation period at 25°C, mouse R2 protein solution was placed on ice and desalted on a BioRad 10-DG column. Desalted sample was then passed through two chromatographic columns using fast protein liquid chromatography (FPLC). First, the sample was applied onto a gel filtration (Superose 6) column using 100 mM potassium phosphate buffer, pH 7.6, containing 5% glycerol, 1 mM PMSF and 2 mM DTT. Second, a strong anion exchange (Mono Q) column was utilized. Mouse R2 protein was eluted with a gradient of 0-0.3 M sodium chloride (NaCl) in 50 mM Tris-HCl, pH 7.6, 5% glycerol, 1 mM PMSF and 2 mM DTT. Last, partially purified protein (~50% purity) was tested for enzyme activity by using the standard ^{3}H-CDP enzymatic assay.

Overexpression and Purification of Human Thioredoxin (Htrx)

An overnight culture of *E. coli* BL21(DE3) carrying pET15b.6xHis-tagged htrx was grown in LB containing 100 μg/ml ampicillin at 37°C with aeration, then inoculated at 1:100 dilution into 1 liter of LB in the presence of ampicillin at the same concentration. The culture was incubated at 37°C until OD_{600} = 0.5-0.6

before IPTG was added at a final concentration of 0.5 mM. After induction was carried out at 37°C for 3.5 hours, bacterial cells were harvested by centrifugation at 4,000 ×g, 4°C for 20 minutes.

The cell pellet was resuspended in 2-5 volumes of 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, placed in liquid nitrogen and stored overnight at -20°C. Frozen cells were slowly thawed in cold water and lysed in a French pressure cell at 15,000 p.s.i. Ribonuclease A and deoxyribonuclease I were then added at a final concentration of 20 μg/ml and 10 μg/ml, respectively and the mixture incubated on ice for 30 minutes. After centrifugation at >10,000 ×g, 4°C, for 20 minutes, supernatant was added to pre-equilibrated Ni-nitrilotriacetic acid (Ni-NTA) agarose resin and incubated on a rotary shaker at 4°C overnight. [Note: Ni-NTA agarose was previously equilibrated in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl containing 5 mM imidazole on a rotary shaker at 4°C for at least 2 hours to prevent non-specific binding.] Ni-NTA agarose resin incubated with supernatant was packed into a 5-ml syringe (Its bottom was plugged with glass fiber filter paper with a stopper attached to the end.). In stepwise fashion, the Ni-NTA column was washed with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl at a flow rate of 0.5 ml/min followed by 50 mM sodium phosphate buffer, pH 6.0, 300 mM NaCl, 10% glycerol containing 5 mM imidazole. After washing the column further with 50 mM sodium phosphate buffer, pH 6.0,

300 mM NaCl, 10% glycerol, human thioredoxin was eluted with 0.1-0.5 M imidazole. Fractions of 1 ml each were collected and analyzed on a 15% polyacrylamide gel.

Ribonucleotide Reductase (RNR) Four-Substrate Assay

RNR Four-substrate assay was performed as previously described (Hendricks and Mathews, 1997; Hendricks and Mathews, 1998a; Hendricks and Mathews, 1998b) with some modifications where specified. On ice, enzymatic reaction mixtures (a total volume of 100 µl each) were set *in vitro* in 50 mM HEPES buffer, pH 8.2, containing 5 mM $MgCl_2$, 50 mM DTT, and 20 µM $Fe(NH_4)_2(SO_4)_2$ with indicated amounts of (deoxy)ribonucleoside triphosphate ((d)NTP) allosteric effectors and ribonucleoside diphosphate (rNDP) substrates. Mouse ribonucleotide reductase large (mR1) and small (mR2) subunits were last added to the reaction mixtures at a final concentration of 1 µM and 2 µM, respectively. After incubation at 37°C for 5 minutes, reactions were stopped by adding 5 µl of 50% perchloric acid (PCA), and the mixtures vortexed, and incubated on ice for 5 minutes. Reaction mixtures were then neutralized by adding 1 N sodium hydroxide (NaOH). Five microliters of 1 M ammonium bicarbonate (NH_4HCO_3) pH 8.9, and 1.5 µl of 0.1 M $MgCl_2$ were subsequently added to each sample. After vortexing, each sample was transferred to a Nanosep-10K centrifugal

device (Pall Gelman Laboratory) and centrifuged at 12,000 rpm (12,500 ×g), 4°C for 6 minutes to remove the enzyme. Centrifugation step was repeated after adding 100 µl of 50 mM NH_4HCO_3, pH 8.9, containing 15 mM $MgCl_2$ (boronate column buffer). The filtrate (200 µl total) was removed and then applied onto a boronate column.

Affi-Gel® 601 boronate affinity gel (Bio-Rad) was used to separate ribonucleotides (unreacted rNDP substrates and ATP) from deoxyribonucleotides (deoxyribonucleoside diphosphate (dNDP) products and dNTP allosteric modifiers). Ribonucleotides, which contain cis-diols, were retained on the column, thus removed from the sample before dNDP products (eluate) were quantitated by high pressure liquid chromatography (HPLC). After a total volume of 200 µl of processed sample was loaded onto a boronate column, another 100 µl of boronate column buffer was applied. The first 300 µl of flowthrough was discarded. Afterward a fraction of 1.8 ml (experimentally shown to yield 93 to nearly 100% recovery) was collected from a boronate column, and its pH adjusted by concentrated phosphoric acid (H_3PO_4) to pH 3-4, following which 500 µl was analyzed by HPLC. A known amount of deoxyribonucleotide standards was also injected (in a separate run) to obtain response factors from which dNDP product amounts were calculated. The variation of the assay is approximately 2 to 3%.

A strong anion exchange column (Whatman Partisil 10 SAX; 4.6 mm× 25 cm) was routinely used with detection at 260 nm. In a 45-min run, the column was equilibrated in 0.075 M monobasic ammonium phosphate ($NH_4H_2PO_4$), pH 3.70, and deoxyribonucleotides were resolved by a biphasic gradient of 0.075 M (Buffer A) to 1 M (Buffer B) $NH_4H_2PO_4$, pH 3.70, at a flow rate of 1.5 ml/min programmed as follows.

Time	%B
0:00	0.0
11:00	21.5
28:30	73.0
29:20	100.0
35:00	100.0
40:00	0.0
45:00	0.0

Anaerobic RNR Four-Substrate Assay

The procedure for anaerobic RNR four-substrate assay was essentially similar to that carried out aerobically. However, reaction components (placed on ice) were assembled inside an anaerobic chamber (equilibrated with an anaerobic gas mixture: 5% H_2, 5% CO_2, and 90% N_2). After 5-min incubation period at 37°C,

reactions were quenched by adding 5 µl of 50% PCA, and the mixtures vortexed and incubated on ice for 5 minutes. Samples were then taken out of the chamber to be processed. Boronate chromatography was performed to remove ribonucleotides, followed by anion-exchange high pressure liquid chromatography for dNDP product formation analysis. In anaerobic preincubation experiments, reaction mixtures and enzyme subunits were gently bubbled with argon gas before being placed in the anaerobic chamber. Mouse R1 and R2 proteins were anaerobically incubated on ice for 20, 40 and 60 minutes before being added to reaction mixtures. Subsequent steps were carried out as previously described. For enzyme regeneration after hydroxyurea treatment, mR2 protein was first incubated with 1 M hydroxyurea at 37°C for 10 minutes in the anaerobic chamber. Hydroxyurea was then removed by using a Centri-Sep spin column (Princeton Separations) before adding mR2 to reaction mixtures at a final concentration of 2 µM. Concentrations of other components—mR1, $MgCl_2$, DTT, and $Fe(NH_4)_2(SO_4)_2$ remained the same. However, all four substrates and allosteric modifiers were present at their estimated physiological concentrations. Regeneration reactions were incubated in the anaerobic chamber at 37°C for 5 minutes. During this period, each sample was gently bubbled with air (20% O_2 to serve as aerobically regenerated control) or with anaerobic gas mixture in the presence of various limited O_2 levels. Reactions were stopped by adding 5 µl of 50% PCA,

and the mixtures vortexed and incubated on ice for 5 minutes before being taken out from the chamber for analysis by boronate chromatography followed by HPLC.

Ultrafiltration Assay for Nucleotide Binding

Commercially available ^{3}H-labeled nucleotides were checked for purity by using thin layer chromatography (TLC). Nucleotide samples were spotted on a polyethyleneimine (PEI) cellulose TLC plate (2 cm from the bottom end of the plate) and air-dried. Separation was carried out first in 1 M lithium chloride (LiCl) for 3 cm, and the chromatogram was washed in methanol for 5 minutes, and air-dried. Samples were then resolved further in 0.8 M LiCl/5% borate, pH 7.0. After 3 hours, the TLC plate was again washed twice (for 5 minutes each time) in methanol and air-dried. Nucleotide spots were detected under a UV lamp, cut off, and eluted with 1 ml (for each spot) of 0.5 N hydrochloric acid (HCl) for 15 minutes. Radioactivity was determined in a scintillation counter followed by calculation of % purity.

$$\% \text{ purity of dNTP} = \left[\frac{[\text{total nucleotide counts} - \text{dNMP counts} - \text{dNDP counts}]}{\text{total nucleotide counts}} \right] \times 100$$

Nucleotide binding assay was performed as previously described by Ormö and Sjöberg (1990). In a 150-µl reaction mixture, the large subunit of mouse RNR and ^{3}H-labeled dNTP were combined in 250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$,

10 mM DTT, and incubated on ice for 15 minutes. A fraction of 30 µl was drawn for scintillation counting, and calculated as total nucleotide concentration. The remaining fraction of 120 µl was transferred to an ultrafree-MC filter unit with modified polyethersulfone (Nanosep-30K MW cut-off; Pall Gelman Laboratory), and centrifuged at 6,000 rpm (3,130 ×g) for 2 min. The filtrate (30 µl) was taken for scintillation counting as free nucleotide concentration. Finally, concentration of bound nucleotides was determined by subtracting free from total nucleotide concentrations.

Measurement of (Deoxy)Ribonucleotide Pools in Mammalian Cells

V79 Cell Culture and Nucleotide Extraction

V79 hamster lung cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; high glucose) containing 5% fetal bovine serum, as previously described (Arecco *et al.*, 1988). Cells were grown as monolayer on 100 mm × 20 mm polystyrene cell culture dishes (Corning®) until nearly 100% confluent. In attempts to change ADP/ATP ratios, cells were treated with varied concentrations of oxidative phosphorylation uncoupling agents—2, 4-dinitrophenol (DNP) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). After incubation at 37°C for 2 hours, cells were monitored for cytotoxicity under a

microscope before culture medium containing uncouplers was removed by aspiration. Cells were then washed twice with ice-cold 1X Tris-buffer saline (TBS; 100 mM Tris-HCl, pH 7.5, 150 mM NaCl). Ice-cold 60% methanol/1% toluene was added before culture dishes were placed at -20°C for 1.5 hours. Methanol/toluene (containing extracted small molecules) was transferred to microcentrifuge tubes. After centrifugation at 15,000 rpm (14,000 ×g), 4°C for 30 minutes, supernatant was transferred to fresh microcentrifuge tubes and dried in a Speed Vac concentrator. Dried samples were resuspended in ice-cold 5% trichloroacetic acid (TCA), and centrifuged again at 5,000 rpm (2,170 ×g), 4°C for 5 minutes, followed by centrifugation at 15,000 rpm (14,000 ×g), 4°C for 5 minutes. Supernatant was transferred to a fresh microcentrifuge tube and extracted by 1.5 volumes of ice-cold Freon/tri-n-octylamine (TOA). After extraction, a quick spin was performed at room temperature at 15,000 rpm (14,000 ×g) to separate two phases. The upper phase containing nucleotides (pH ≈ 5.5-6.0) was transferred to a fresh microcentrifuge tube, and dried in a Speed Vac concentrator. Dried extract was kept at –20°C for nucleotide level measurements.

Determination of rNDPs and rNTPs by Using HPLC

Each dried extract (obtained from 3 plates of cells) was resuspended in 300 µl of distilled water (dH_2O) and divided into 3 parts. To the first 100-µl portion, 400 µl of 0.075 M $NH_4H_2PO_4$, pH 3.70 (HPLC buffer A), was added, followed by adjustment to pH 3-4 with concentrated H_3PO_4. This part was then directly injected to the HPLC for total nucleotide measurement. Another 100 µl was for determination of deoxyribonucleotides. One hundred microliters of boronate column buffer (50 mM NH_4HCO_3 pH 8.9, containing 15 mM $MgCl_2$) was added before applying the extract to a boronate column. Deoxyribonucleotides were eluted according to a standard procedure for boronate chromatography (1.8 ml), and dried in a Speed Vac concentrator until the total volume was $\leq$ 500 µl. If necessary, the volume was made up to 500 µl with HPLC buffer A before adjusting pH to 3-4 with H_3PO_4. The sample was then analyzed by HPLC. The amount of ribonucleotides was achieved by the difference between total nucleotides and deoxyribonucleotides. The last fraction of dried extract was saved for determination of dNTP levels by dNTP pool enzymatic assay, as described in the next section.

Determination of dNTPs by Using dNTP Pool Enzymatic Assay

An enzymatic assay for measuring dNTP pools was one of the standard methods developed in this laboratory. A solution of 5% TCA/2% sodium pyrophosphate (PP_i) was freshly prepared each time the assay was performed. This solution (100 µl per each 2 cm × 2 cm square) was added onto squares of Whatman 3 MM chromatography paper (number of squares corresponded to number of samples in duplicates) before allowing the squares to dry under an infrared lamp. On ice, a reaction mixture containing 5X dNTP buffer (250 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$) , poly dIdC: poly dIdC template (for dGTP and dCTP pools) or poly dAdT: poly dAdT template (for dATP and dTTP pools), complementary dNTP, ^{3}H-labeled complementary dNTP, dAMP, bovine serum albumin (BSA), DTT, sterile H_2O, with Klenow fragment last added was prepared. Forty-five microliters of reaction mixture was added to each aliquot of nucleotide extracts. After vortexing followed by brief centrifugation, each reaction mixture was incubated in a 37°C water bath for a period of time determined by a standard curve reaction experiment (usually for 45, 60 or 90 minutes). At the end of incubation periods, 20 µl of each reaction mixture was spotted on 5% TCA/2% PP_i pre-equilibrated Whatman 3 MM chromatography paper, and let dry under an infrared lamp again. After sequentially washing 3 times with 5% TCA/2% PP_i and twice with 95% ethanol (15 minutes per wash) and air-drying, each dried filter paper square was placed into a scintillation vial with some scintillation cocktail

and counted for ^{3}H for 5 minutes. Counts per minutes (cpm) of each sample was then converted to picomoles (pmol) of dNTP per 10^{6} cells by using a standard curve obtained from a separate assay performed in the presence of known amounts of standard dNTPs.

Chapter 4

Allosteric Regulation of Mammalian Ribonucleoside Diphosphate Reductase by Nucleoside Triphosphates Analyzed by The Four-Substrate Assay

The four-substrate assay, developed in this laboratory, has been used successfully to study allosteric regulation of ribonucleotide reductase (RNR) from bacteriophage T4, *E. coli* and vaccinia virus (Hendricks and Mathews, 1997; Hendricks and Mathews, 1998). These enzymes are Class Ia ribonucleotide reductases that function aerobically, and contain two allosteric sites and the catalytic site on their large subunits (R1) which bind to nucleoside triphosphate modifiers and ribonucleoside diphosphate (rNDP) substrates, respectively. Since this type of RNR carries four reductase activities, converting rCDP, rUDP, rADP and rGDP to their corresponding deoxyribonucleoside diphosphates, the four-substrate assay is by far the most powerful tool for simultaneous monitoring of the reduction of all four substrates. Although all reaction components are assembled *in vitro*, reaction conditions can simply be adjusted to resemble physiological environments to which the enzyme is exposed inside the cell. Not only is this technique useful for investigating allosteric regulation under aerobic conditions, control under anaerobic conditions has been as well demonstrated (Andersson *et al.*, 2000; Chimploy *et al.*, 2000 (Chapter 6 of this

thesis)). Here, the four-substrate assay was used to examine allosteric regulation of mouse ribonucleotide reductase (Chimploy, 2001), which represented a mammalian enzyme. It was of interest to investigate effects of nucleoside triphosphates upon the enzyme specificity under conditions involving proportional rNDP substrates and allosteric modifiers, simulating physiological environments. Furthermore, by using this technique, it was feasible to monitor changes in rates of product formation influenced by variations in dNTP levels, which could not possibly be accomplished by using the traditional single-substrate assay.

Purification of Mammalian Ribonucleotide Reductase

Recombinant proteins were overexpressed (Figure 4.1), purified, and then tested separately for their activity. Mouse R1 protein was purified to high purity (>95%) by using a dATP-Sepharose column (Figure 4.2). The protein was effectively eluted by 75 mM ATP; however, washing with 5 mM dATP at the final step yielded much more protein, suggesting higher affinity of the enzyme for dATP. Both ATP and dATP were removed from the R1 protein before being used in the four-substrate assay. ATP was replaced with an excessive amount of buffer for R1 purification on a Centricon-30 concentrating device. Separation of dATP was carried out by incubating the R1 protein with 1 M NaCl on ice. After 15 minutes,

a Centri-Sep spin column (Princeton Separations) was used to remove both dATP and NaCl. The amount of ATP or dATP before and after the procedures was quantitated by HPLC. By using these methods, nearly 100% of ATP and up to 99.8% of dATP were successfully eliminated (Figure 4.3). Mouse R2, on the other hand, was only partially purified (~50% purity), after several purification steps (Figure 4.5). Essentially, by using Western blotting (detected by anti-vaccinia virus R2 antibody), three bands with molecular weights of ~43-45 kDa were proven to be the mouse R2 protein (~45 kDa) with corresponding degradation products (~43-44 kDa) (KC RNR Notebook#5, page 29). The antibody did not react with a single band with a molecular weight of ~30 kDa, indicating a major contaminant. Several attempts to separate this particular contaminating protein from the mouse R2 protein were unsuccessful, suggesting that the contaminant may be associated with the protein of interest. Besides, separation of proteins with minor difference in molecular weights was quite a challenge. Nevertheless, ^{3}H-CDP enzymatic assay performed in the absence of mouse R1 showed no evidence of contamination by the E. *coli* host's RNR (Table 4.1). In addition, after a 5-minute incubation period, it was proven to be free of nucleoside diphosphate kinase (NDPK) activity, which could convert dNDP products to their corresponding dNTPs (Table 4.2).

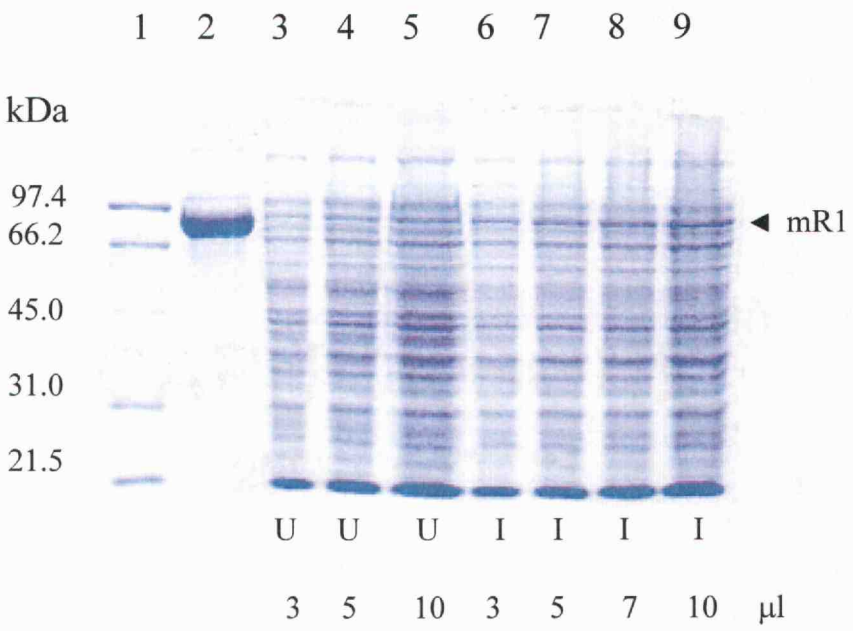


Figure 4.1 Overexpression of recombinant mouse R1 protein. Cell lysates of *E.coli* (BL21/DE3 pLys S) containing mR1 gene inserted into a pET vector system were analyzed on a 10% polyacrylamide gel. Lane 1: low-molecular weight protein markers; Lane 2: 16 µg mR1 protein; Lane 3-5: varied loaded amounts of the uninduced control (U); Lane 6-9: varied loaded amounts of IPTG-induced cells (I). Molecular weight of mR1 protein is ~90 kDa.

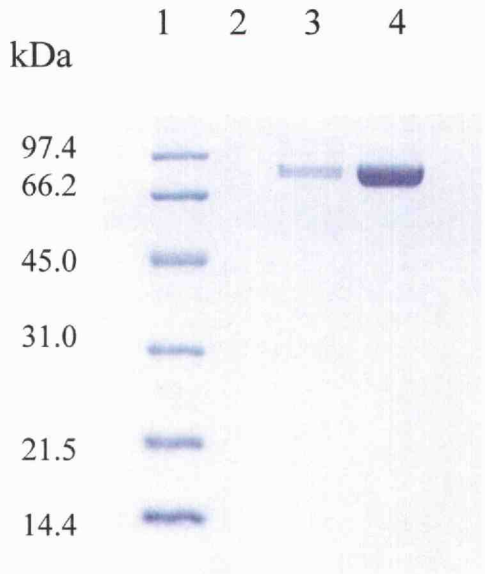


Figure 4.2 Purification of recombinant mouse R1 protein. Purified mouse R1 protein was analyzed on a 10% polyacrylamide gel. Lane 1: low-molecular weight protein markers; Lane 2: none; Lane 3: purified mR1 in a 75-mM ATP fraction; Lane 4: purified mR1 in a 5-mM dATP fraction.

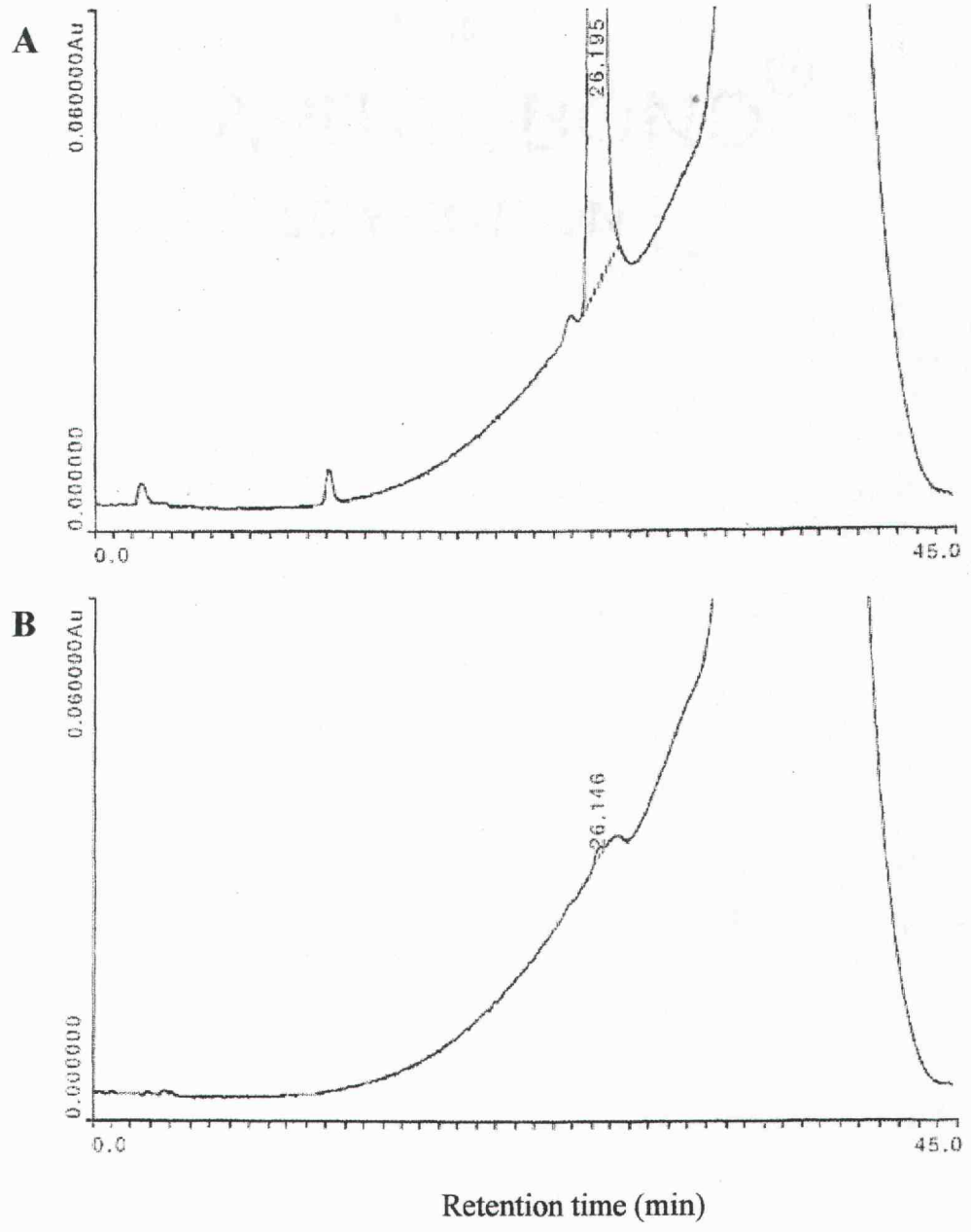


Figure 4.3 Separation of dATP from the mouse R1 protein by a spin column after NaCl treatment. The mR1 protein was treated with 1 M NaCl for 15 minutes on ice. The salt-treated protein was then applied onto a spin column (3,000 rpm for 2 minutes). The amount of dATP in protein fractions (5 µl each) before (A) and after (B) NaCl treatment was analyzed by HPLC.

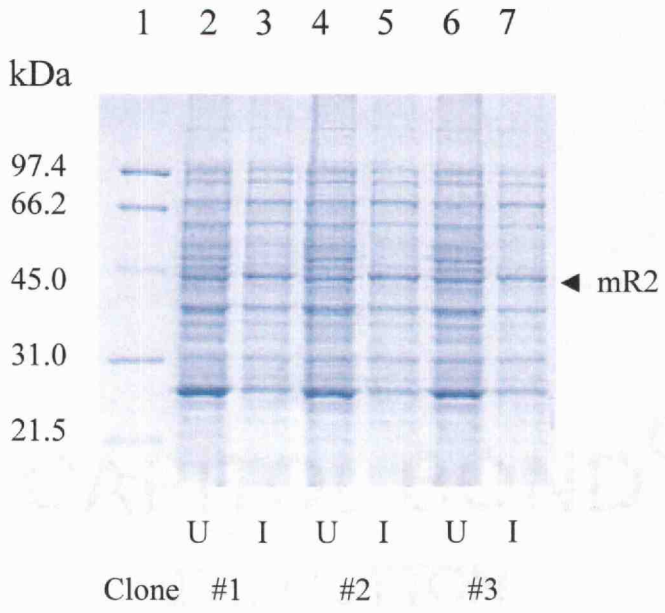


Figure 4.4 Overexpression of recombinant mouse R2 protein. Cell lysates of *E.coli* (BL21/DE3 pLys S) containing mR2 gene inserted into a pET vector system were analyzed on a 12% polyacrylamide gel. Lane 1: low-molecular weight protein markers; Lane 2: uninduced (U) control#1; Lane 3: induced (I) lysate#1; Lane 4: uninduced control#2; Lane 5: induced lysate#2; Lane 6: uninduced control#3; Lane 7: induced lysate#3. Molecular weight of mR1 protein is ~45 kDa.

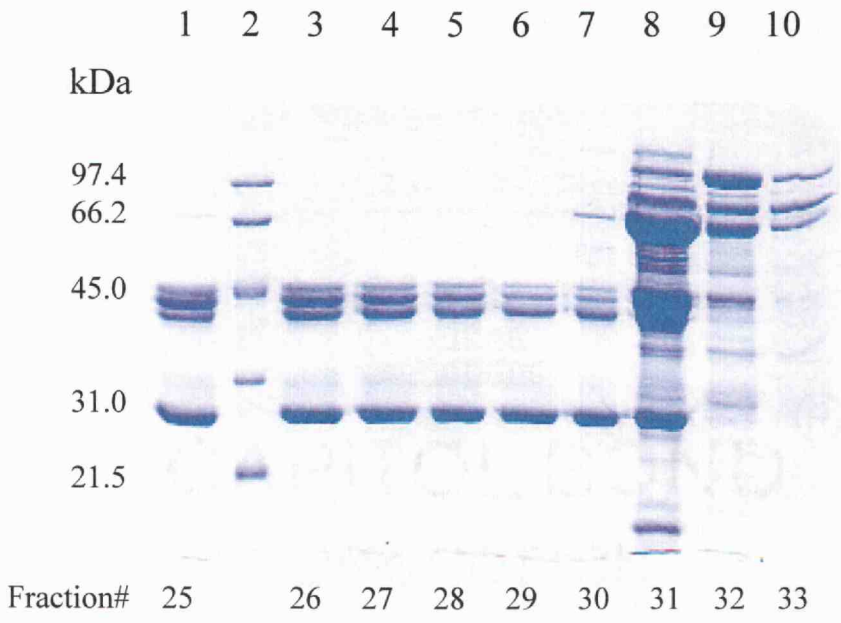


Figure 4.5 Purification of recombinant mouse R2 protein. Partially purified mouse R2 protein (estimated to be ~50% pure) was analyzed on a 12% polyacrylamide gel after MonoQ anion-exchange chromatography. The protein was eluted with a gradient of 0-0.3 M NaCl in 50 mM Tris-HCl,pH 7.6, containing 2 mM DTT, 1 mM PMSF and 5% glycerol. Lane 1: fraction#25, Lane 2: low-molecular weight protein markers; Lane 3-10: fraction#26-33. Only fractions#25 to #29 were pooled and used in the four-substrate assay. The three bands are the full-length (~45 kDa) mouse R2 protein with corresponding degradation products (~43-44 kDa) determined by Western blotting. The major contaminant has a molecular weight of ~30 kDa.

Table 4.1 Ribonucleotide reductase activity of freshly purified mR1 and mR2 proteins determined by ^{3}H-CDP enzymatic assay. A"Blank" reaction mixture containing no protein and reaction mixtures containing mR1 and/or mR2 were incubated with ^{3}H-CDP substrate at 37°C for 60 minutes before reactions were stopped by PCA prior to analysis of ^{3}H-dCDP product formation by TLC (thin layer chromatography). Old mR1 and mR2 proteins (known to be active) were previously purified in the lab, and hence were included as a positive control to validate the assay. Freshly purified (New mR1 and mR2) subunits, however, were ones being used throughout the entire project. Data are presented as "Counts per minute" (cpm) and "Specific activity" (pmol dCDP/mg/hr).

Reaction conditions	Counts per minute (cpm)	Specific activity (pmol dCDP/mg/hr)
Background (no proteins added)	105	0.00
Positive control (old mR1+old mR2)	21957	263.84
New mR1 only	184	25.80
New mR2 only	156	1.28
New mR1+new mR2	20938	485.11

Table 4.2 Nucleoside diphosphate kinase activity determination in preparation of mR2 protein. dCDP was incubated with mR2 in 50 mM HEPES, pH 8.2, containing 20 mM $MgCl_2$, 50 mM DTT and 2.5 mM ATP at 37°C for 5, 10 and 20 minutes. Reactions were stopped and mixtures processed as previously described under the four-substrate assay.

Time of incubation (minutes)	Percentage of total product		
	dCMP*	dCDP	dCTP
5	<0.1	100	<0.1
10	0.6	98	1.4
20	0.9	98	1.1

* Trace amount of dCMP was possibly due to degradation of dCDP.

Optimal Conditions for the Four-Substrate Assay

Determination of Optimal pH for Mouse Ribonucleotide Reductase Activity

To perform the four-substrate assay, the reaction mixture typically comprised 50 mM HEPES buffer, pH 8.2, 5 mM $MgCl_2$, 50 mM DTT, 20 μM $Fe(NH_4)_2(SO_4)_2$, rNDPs, and (d)NTPs. The R1 and R2 proteins were always added last. The assay was usually carried out at pH 8.2 based on the previous determination of the pH optima for RNRs from vaccinia virus and cultured monkey

kidney cells (B-SC 40) (Slabaugh and Mathews, 1984). As shown in Figure 4.6, when mouse RNR was subjected to an assay under estimated physiological conditions at pH 7.6, the overall product profile was essentially the same as that obtained at pH 8.2. However, its total activity was decreased approximately 25% and 39% when reaction mixtures were incubated at 37°C for 5 and 10 minutes, respectively, suggesting that pH 8.2 was favorable for mouse RNR.

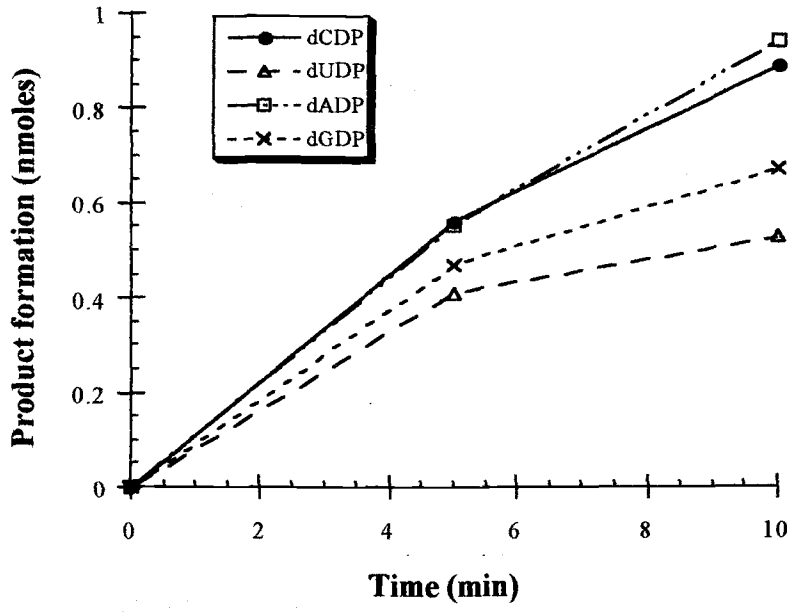
Dithiothreitol versus Thioredoxin as a Reductant

In this system, DTT was routinely used as a reducing agent. Nonetheless, it was interesting to determine whether DTT functioned in the same manner as reductants found *in vivo* such as thioredoxin or glutaredoxin. Because of its availability and high homology to the mouse counterpart, human thioredoxin was an excellent candidate for this experiment. Therefore, it was purified to >95% purity (Figure 4.7), and subjected to the four-substrate assay in order for side by side comparison with the assay carried out in the presence of DTT. Since human thioredoxin was aerobically purified, and hence, likely to be naturally oxidized, it was first reduced by a 15-fold excess of DTT with a 30-minute incubation period at 37°C. Then it was added to the reaction mixture immediately after DTT had been removed by a Centri-Sep spin column. Resazurin dye ($C_{12}H_6NO_4Na$), whose molecular weight (251.2) was slightly greater than that of DTT, was used as

an indicator of complete removal of DTT. Figure 4.8 indicates that product profiles obtained from the two experiments were essentially the same (i.e. dADP>dUDP>dCDP>dGDP) with the least difference in the amount of products formed at 5-minute time point (the usual incubation period for the four-substrate assay). In comparing percentage of total activity in the two experiments, CDP reductase activity remained the same throughout 15 minutes of reaction time, whereas activities of ADP and UDP reductases were slightly higher in the presence of human thioredoxin as a reducing agent. Interestingly, GDP reductase was more active in the presence of DTT (Table 4.3). In fact, formation of dGDP in the presence of human thioredoxin was approximately two-fold lower than that in the presence of DTT, suggesting different mechanisms by which the two reductants influence GDP reductase activity of mouse RNR.

Figure 4.6 pH dependence of mouse ribonucleotide reductase activities. The four-substrate assay was performed at pH 7.6 (A) and 8.2 (B) in the presence of estimated *in vivo* concentrations of the four rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP, and 160 μM UDP) and allosteric effectors (2.5 mM ATP, 60 μM dATP, 15 μM dGTP, and 50 μM dTTP). At each pH, reaction mixtures were incubated at 37°C for 0, 5 and 10 minutes.

A


dCDP
dUDP
dADP
dGDP
Product formation (nmoles)
Time (min)
0
0.2
0.4
0.6
0.8
1
0
2
4
6
8
10

B

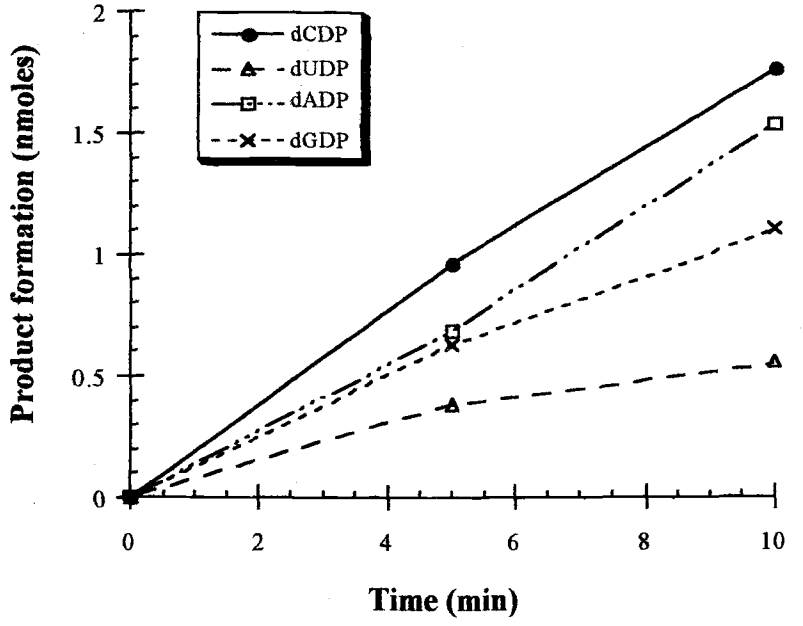

dCDP
dUDP
dADP
dGDP
Product formation (nmoles)
Time (min)
0
0.5
1
1.5
2
0
2
4
6
8
10

Figure 4.6

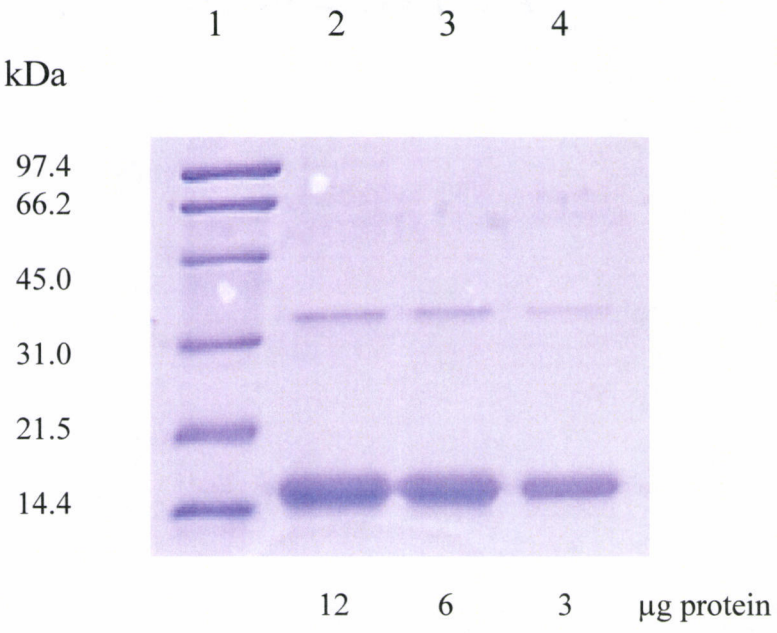


Figure 4.7 Purification of human thioredoxin. Varied loading amounts of purified human thioredoxin, eluted by 0.1-0.3 M imidazole are shown in lane 2-4. Compared to the standard protein markers in lane 1, monomer thioredoxin has a molecular mass of ~12-14 kDa. A minor band in each lane at ~30 kDa corresponds to dimer formation of thioredoxin protein.

Figure 4.8 Effects of dithiothreitol and human thioredoxin upon the four activities of mouse ribonucleotide reductase. The four-substrate assay was carried out in the presence of 50 mM DTT (A) and 100 μM human thioredoxin (B). Both reaction mixtures contained 1 and 2 μM of mR1 and mR2, respectively, and rNDP and allosteric effectors at their quasi-physiological concentrations. Incubation periods were varied as indicated.

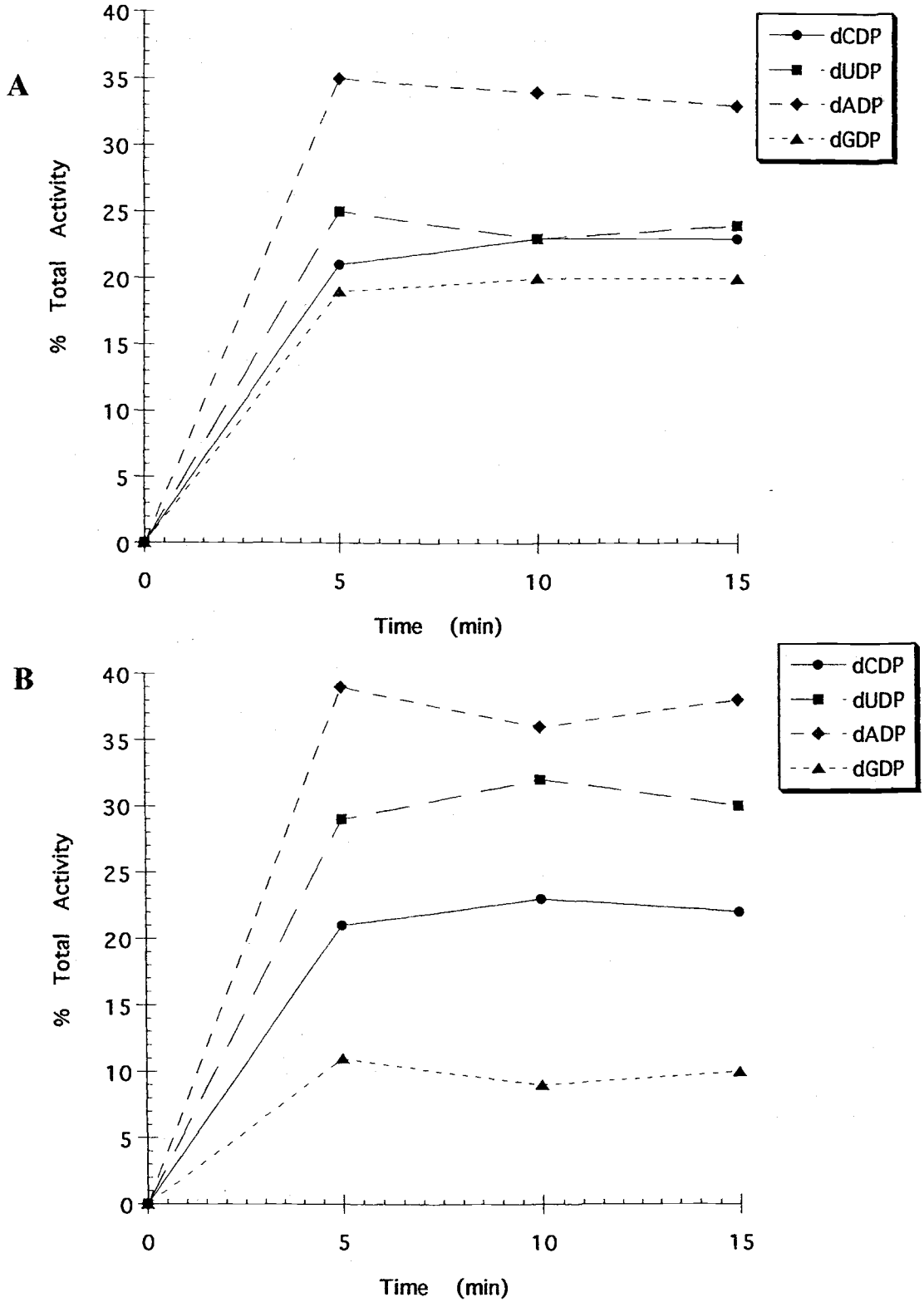

A
% Total Activity
40
35
30
25
20
15
10
5
0
0
5
10
15
Time (min)
dCDP
dUDP
dADP
dGDP
B
% Total Activity
40
35
30
25
20
15
10
5
0
0
5
10
15
Time (min)
dCDP
dUDP
dADP
dGDP

Figure 4.8

Table 4.3 A comparison of effects of DTT and human thioredoxin upon mouse ribonucleotide reductase specificity. The four-substrate assay was carried out under quasi-physiological conditions. Mouse R1 and R2 proteins were present at final concentrations of 1 and 2 μM, respectively. Reaction mixtures containing either 50 mM DTT or 100 μM human thioredoxin were incubated at 37°C for 0, 5, 10 and 15 minutes before being stopped by 50% PCA and processed as described in Materials and Methods.

		Percentage of total activity			
Reductant	Time of incubation (minutes)	dCDP	dUDP	dADP	dGDP
Dithiothreitol	0	0	0	0	0
	5	21	25	35	19
	10	23	23	34	20
	15	23	24	33	20
Thioredoxin	0	0	0	0	0
	5	21	29	39	11
	10	23	32	36	9
	15	22	30	38	10

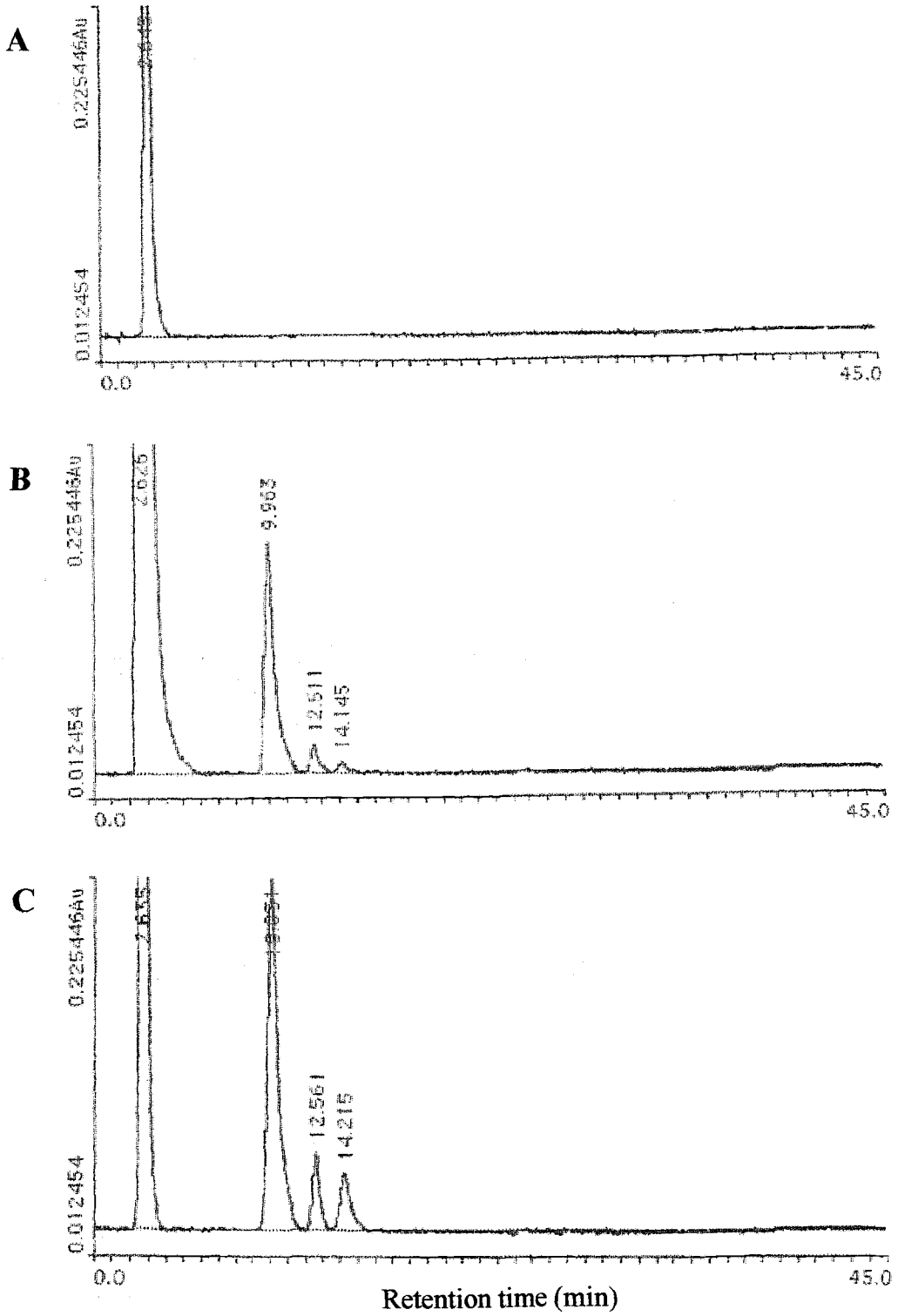
Effect of ATP versus AMP-PNP as a Primary Activator

For Class Ia RNRs, ATP binds to two allosteric sites—the activity site and specificity site, affecting the enzyme's overall activity and substrate specificity, respectively. ATP has been shown to be a universal activator for RNRs in many organisms. Here, this was proven true for mouse RNR, a representative of mammalian RNRs, as shown in Figure 4.9. Additionally, at least for mouse RNR,

all four activities were negligible when the four-substrate assay was carried out in the absence of ATP or dNTP effectors (Figure 4.10).

An ATP analog, 5'-adenylylimidodiphosphate (AMP-PNP), could also act as a primary activator. However, it did not stimulate the enzyme in the same manner as ATP did since a different product profile was observed (Figure 4.11), consistent with what was observed with vaccinia virus RNR (Hendricks Ph.D. thesis, 1998). Table 4.4 compares percentage of product formation in the assays performed in the presence of ATP or AMP-PNP as a sole allosteric modifier, and equimolar substrates. Whereas reduction of CDP was comparable, activation of UDP and ADP reduction by AMP-PNP was two- and four-fold, respectively lower than that by ATP. Interestingly, GDP reduction did not require dTTP when AMP-PNP was present. Moreover, the fact that ADP formation was almost negligible was in an agreement with the assays conducted with *E. coli* and vaccinia virus RNRs (Hendricks Ph.D. thesis, 1998). When both rNDP substrates and allosteric effectors (with ATP replaced by AMP-PNP) were present at their estimated physiological concentrations, nonetheless, the product profile became more similar to that obtained in the presence of ATP. In fact, it was also closer to the nucleotide composition of the mouse genome (21% C, 29% T, 29% A, and 21% G) (Table 4.5).

Figure 4.9 ATP as a general activator. The reaction mixture contained 1 μM mR1, 2 μM mR2, 0.15 mM each of rNDP substrates and 2.5 mM ATP as a single activator. The reaction mixtures were incubated at 37°C for 0 (A), 10 (B) and 20 (C) minutes.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45.0
B
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0.012454
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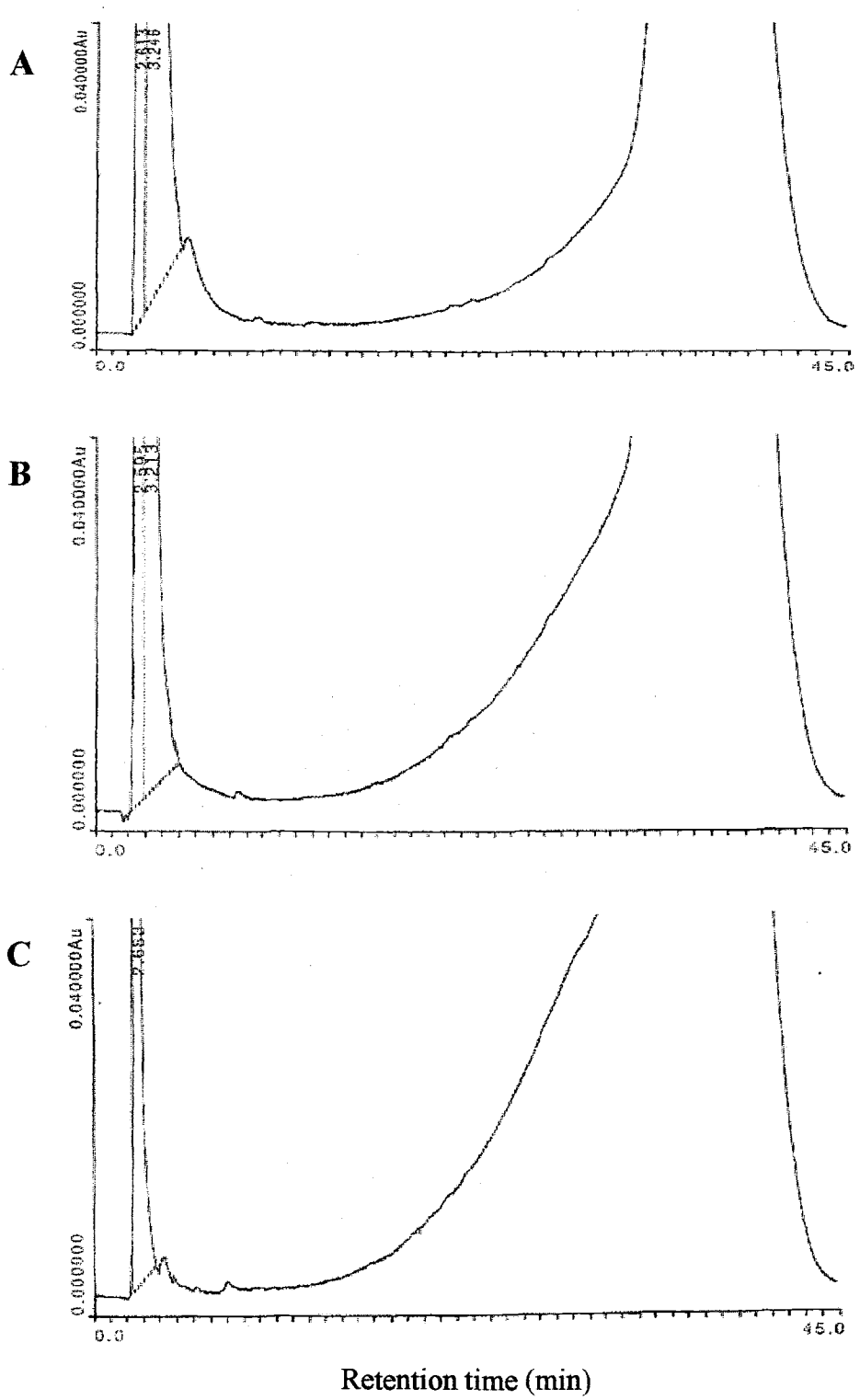
12.511
14.145
0.0
45.0
C
0.225446Au
0.012454
2.675
12.561
14.215
0.0
45.0
Retention time (min)

Figure 4.9

Figure 4.10 ATP dependence of mouse ribonucleotide reductase. The four-substrate assay was performed in HEPES buffer, pH 8.2, containing 5mM $MgCl_2$, 50 mM DTT and 20 μM $Fe(NH_4)_2(SO_4)_2$ in the presence of 1 μM mR1, 2 μM mR2, 0.15 mM each of rNDP substrates. In this experiment, no ATP or dNTP was added. The reactions were carried out at 37°C for 0 (A), 5 (B) and 10 (C) minutes.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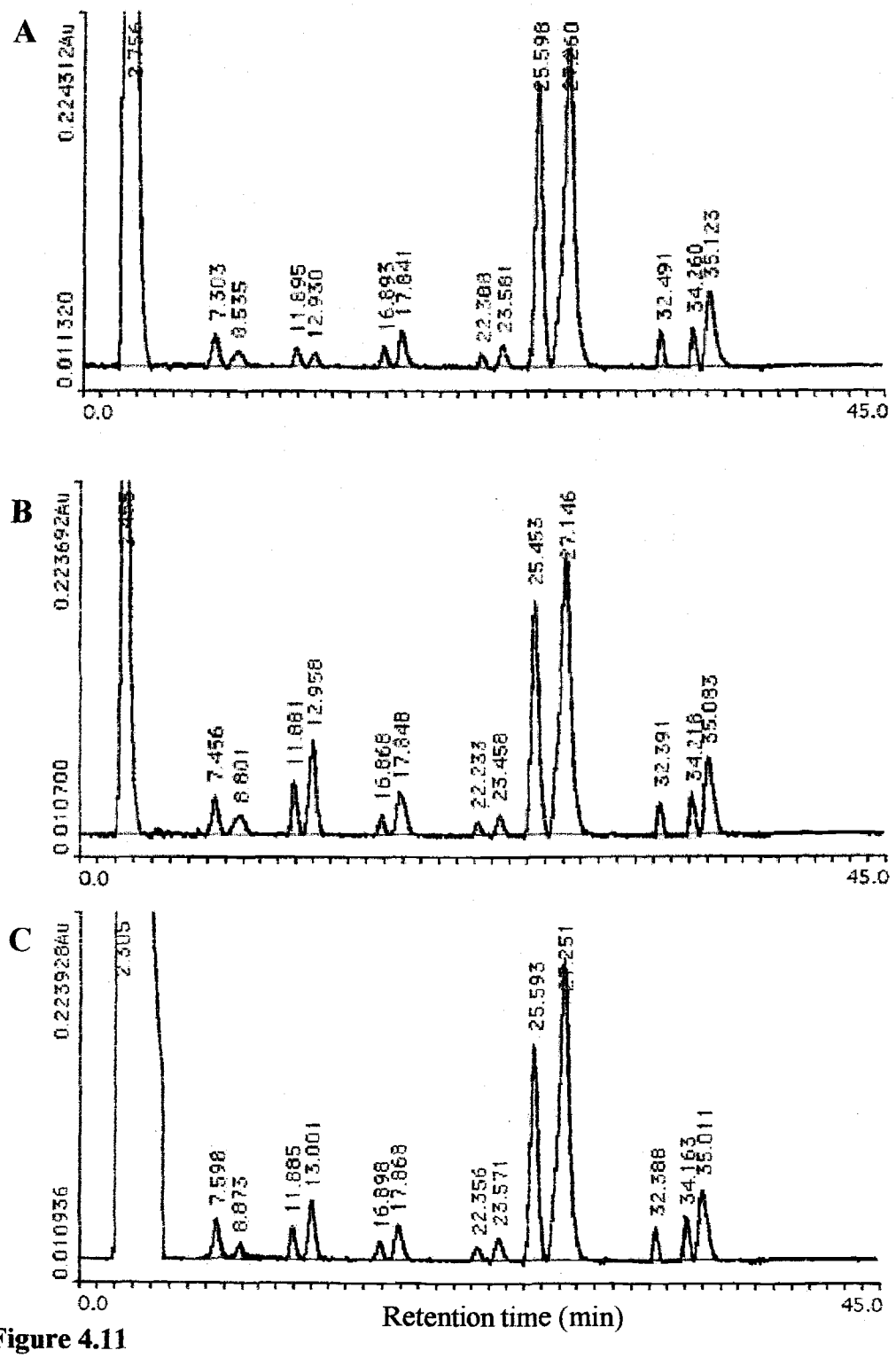
45.0
B
0.040000Au
0.000000
0.0
45.0
C
0.040000Au
0.000000
0.0
45.0
Retention time (min)

Figure 4.10

Figure 4.11 Effect of an ATP analog, 5'-adenylylimidodiphosphate (AMP-PNP) on mouse ribonucleotide reductase activities. This HPLC chromatogram shows reduction of all four rNDP substrates by mouse RNR in the presence of AMP-PNP as a primary activator. The reaction mixture contained 1 μM mR1, 2 μM mR2, quasi-physiological concentrations of rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP) and allosteric modifiers (60 μM dATP, 15 μM dGTP and 50 μM dTTP). AMP-PNP was added, instead of ATP at a final concentration of 2.5 mM. The reaction mixtures were incubated at 37°C for 0 (A), 5 (B) and 10 (C) minutes.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2.756
7.303
8.535
11.895
12.930
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17.841
22.388
23.561
25.598
32.491
34.260
35.123
0.0
45.0
B
0.223692AU
0.010700
7.456
8.801
11.881
12.958
16.868
17.848
22.233
23.458
25.453
27.146
32.391
34.216
35.083
0.0
45.0
C
0.223928AU
0.010936
2.305
7.598
8.873
11.885
13.001
16.898
17.868
22.356
23.571
25.593
32.388
34.163
35.011
0.0
45.0
Retention time (min)

Figure 4.11

Table 4.4 Effect upon mouse ribonucleotide reductase specificity of AMP-PNP. Mouse R1 and R2 proteins were present at final concentrations of 1 and 2 μM, respectively. Reaction mixtures contained four rNDP substrates at 0.15 mM each and only ATP or AMP-PNP as the enzyme activator.

	Percentage of total product formed			
Assay conditions	dCDP	dUDP	dADP	dGDP
ATP only	78	18	4	0
AMP-PNP only	87	8	1	4

Table 4.5 Effect upon mouse ribonucleotide reductase specificity of AMP-PNP under quasi-physiological conditions. Mouse R1 and R2 proteins were present at final concentrations of 1 and 2 μM, respectively. Estimated *in vivo* concentrations of the four rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP, and 160 μM UDP) and allosteric effectors (2.5 mM ATP, 60 μM dATP, 15 μM dGTP, and 50 μM dTTP) were included in the reaction mixtures. Percentage of total product formed, in the presence of AMP-PNP, is presented as averaged values obtained from two independent experiments ± standard deviation.

	Percentage of total product formed			
Assay conditions	dCDP	dUDP	dADP	dGDP
ATP	34	25	27	15
AMP-PNP	17±5.66	24±1.41	39±4.95	21±0.71

Effects of Allosteric Modifiers upon Mouse Ribonucleotide Reductase Specificity

In order to understand a specific role of each allosteric regulator as bound to the specificity site on the large subunit of mouse RNR, the four-substrate assay was performed at constant concentrations of ATP and individual dNTPs. Chromatograms demonstrating product profiles and corresponding numerical values are illustrated in Figure 4.12 and Table 4.6, respectively. ATP activated reduction of CDP, UDP, and ADP (Figure 4.12 panel A). Surprisingly, GDP reductase was reproducibly undetectable under this condition. Compared to

Figure 4.12 panel C, reduction of GDP strictly required dTTP. Additionally to its role in primarily activating GDP reduction, dTTP also activated ADP reduction (although to a lesser extent). However, it inhibited reduction of pyrimidine nucleotides (CDP and UDP). dCTP showed no effect on mouse RNR specificity (Figure 4.12 panel B), consistent with previous studies in a single-substrate assay (Reichard *et al.*, 2000) and the four-substrate assay performed on T4 phage and vaccinia virus RNRs (Hendricks and Mathews, 1997; Hendricks and Mathews, 1998). dGTP strongly activated ADP reduction and, to a lesser extent, GDP reduction while inhibiting reduction of CDP and UDP (Figure 4.12 panel E). At a concentration of 40 μM, dATP slightly activated ADP reduction, whereas it inhibited reduction of other nucleotides (Figure 4.12 panel D), indicating its significant binding to the activity site.

Figures 4.13 and 4.14 depict the inhibitory effects of dATP on total activity of mouse RNR. In Figure 4.13, the enzyme was assayed in the presence of equimolar rNDP substrates (0.15 mM), 2.5 mM ATP and indicated amounts of dATP. By contrast, in Figure 4.14, the assay was performed under quasi-physiological conditions (substrates: 850 μM ADP, 70 μM CDP, 160 μM GDP, 160 μM UDP; effectors: 2.5 mM ATP, 15 μM dGTP, 50 μM dTTP, and varied amounts of dATP). As shown in both figures, the mouse RNR was inhibited significantly by dATP. In the presence of 20 μM dATP, 75-78% of the potential reducing activity was repressed. At the estimated *in vivo* concentration of dATP

(60 μM), only 8 (equimolar substrates +ATP only) to 16 ("*in vivo*" conditions) % of total activity remained, indicating a tight control by dATP inside the cell. As the concentrations of dATP increased, the total activity of the enzyme continued to drop when ATP was the only other regulator, with a tendency to approach 0% remaining activity (Figure 4.13). However, when the other dNTPs were also present, the enzyme activity remained constant at approximately 14-16%, suggesting a lower sensitivity to dATP due to a balanced control by other allosteric modifiers (Figure 4.14).

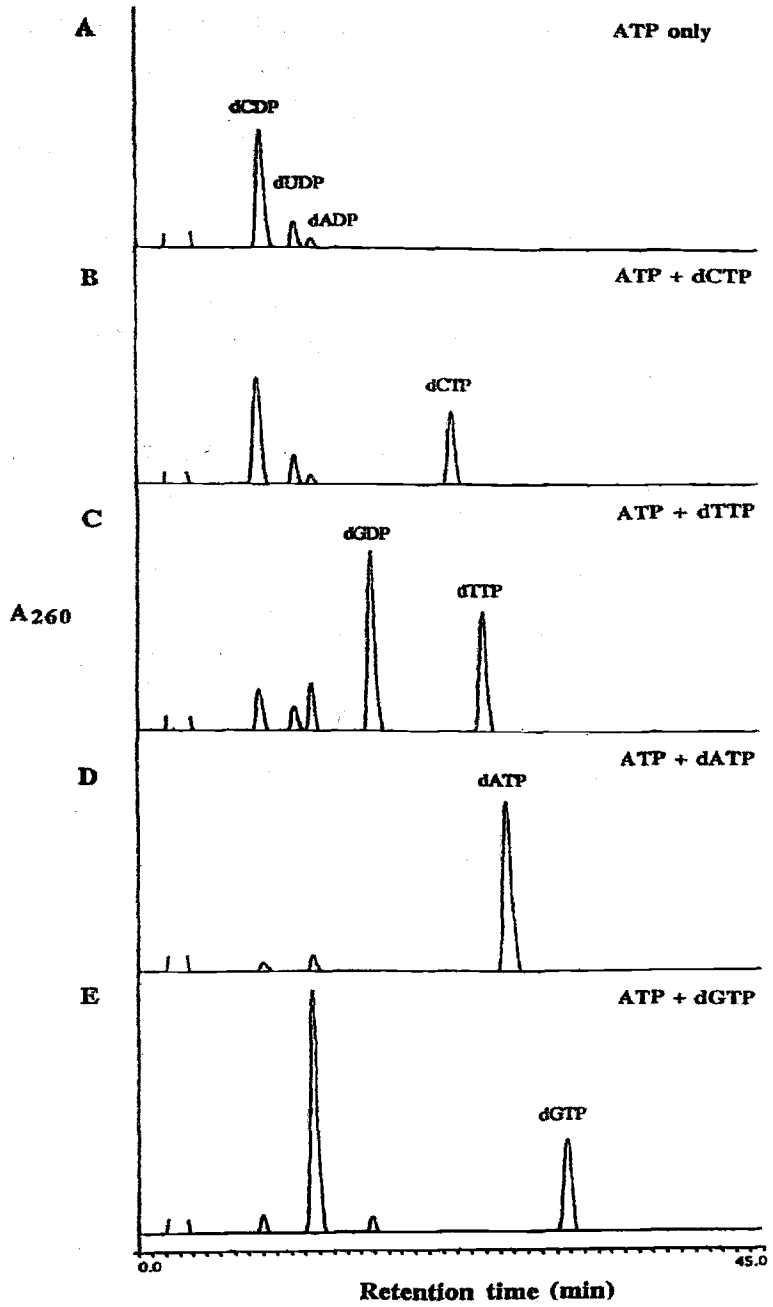


Figure 4.12 Effects of individual dNTP allosteric modifiers upon mouse ribonucleotide reductase specificity analyzed by the four-substrate assay. All reaction mixtures contained the four rNDP substrates at equimolar (0.15 mM) and 2.5 mM ATP. Each dNTP, where indicated, was added at a final concentration of 40 μM.

Table 4.6 Effects upon mouse ribonucleotide reductase activities of individual dNTP allosteric regulators. Each reaction mixture containing 0.15 mM rNDP substrates was incubated at 37°C for 5 minutes. ATP and each dNTP were present at final concentrations of 2.5 mM and 40 μM, respectively.

dNTP effector	Rate of product formation (nmol dNDP/min/mg R1 protein)			
	dCDP	dUDP	dADP	dGDP
None	108.0	11.1	2.4	<0.1
dCTP	108.2	11.3	2.4	<0.1
dTTP	31.8	10.9	13.3	78.4
dATP	7.6	<0.1	5.1	<0.1
dGTP	11.1	<0.1	92.7	5.3

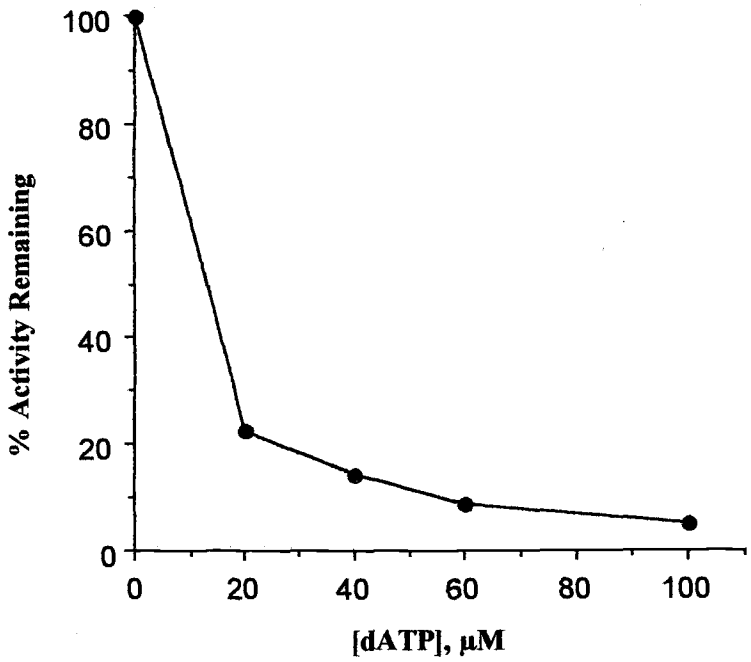


Figure 4.13 Inhibition of the mouse RNR by dATP. The assay was performed in the presence of 1 μM mR1, 2 μM mR2, 1.5 mM each of rNDPs, 2.5 mM ATP, and indicated amounts of dATP. "% Activity Remaining" refers to the enzyme activity that remains after addition of dATP compared to the activity measured in the presence of 2.5 mM ATP only.

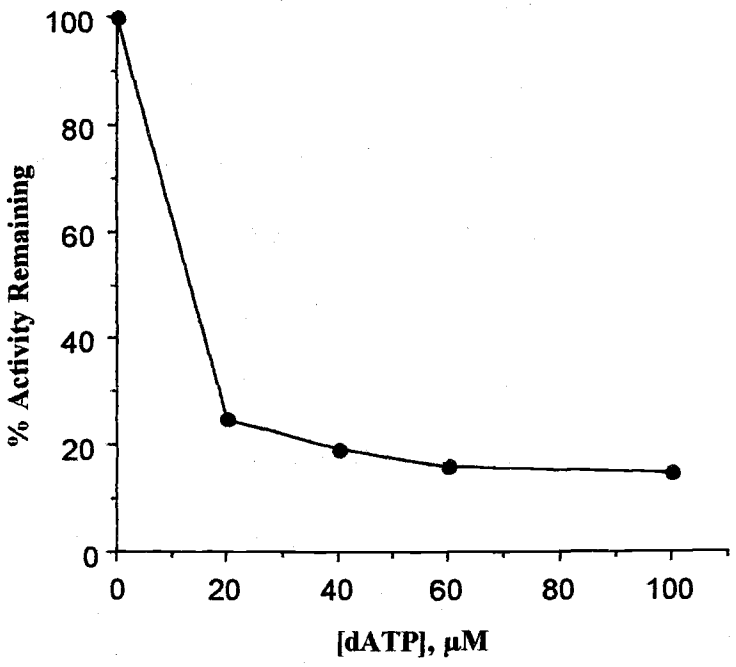


Figure 4.14 Inhibition of the mouse RNR by dATP under estimated *in vivo* conditions. In this experiment, the rNDP substrates and allosteric effectors were present at their estimated physiological concentrations with varied amounts of dATP. The remaining activity was calculated the same way as that in Figure 4.13.

Proportional Variations in dNTP Concentration

It was of interest to simultaneously examine effects of all four nucleotide modifiers in one reaction in order to understand allosteric regulation under conditions to which the enzyme was exposed. To this end the four-substrate assay was performed in the presence of approximate physiological concentrations of rNDP substrates and proportionally varied concentrations of allosteric effectors. As illustrated in Figure 4.15, allosteric regulators differentially affected the four activities of the enzyme. When they were present at below their estimated *in vivo* concentrations, reduction of ADP, GDP and UDP were slightly activated, whereas CDP reduction was nearly 2-fold inhibited. In fact, a decrease of CDP formation continued as the added amount of effector mixture was increased. Formation of ADP, GDP and UDP, on the other hand, seemingly remained constant once concentrations of modifiers were beyond their approximate physiological concentrations. In addition to differential effects upon enzyme specificity of modifiers, the product profile (the order of activities being A > C > G > U) at the point where estimated *in vivo* concentration of both substrates and effectors were present was the same as when the substrates were bioproportionally varied (Figure 5.1, Chapter 5), indicating consistency of the assays.

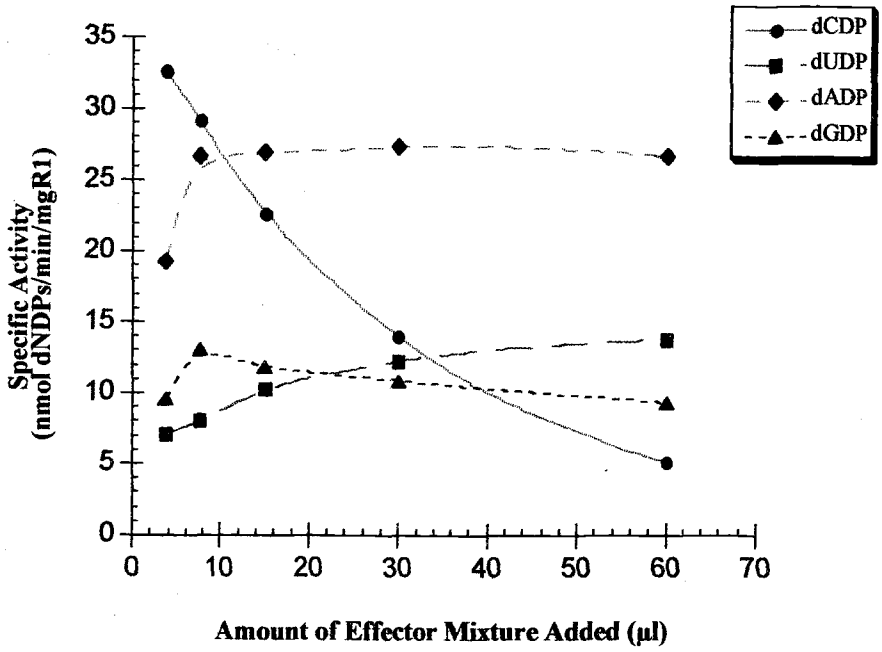


Figure 4.15 Effects of proportional variations in allosteric modifiers upon the four activities of mouse ribonucleotide reductase. The four-substrate assay was carried out in the presence of 1 µM mR1, 2 µM mR2 with "*in vivo*" concentrations of rNDPs (850 µM ADP, 70 µM CDP, 160 µM GDP and 160 µM UDP). Allosteric effectors were proportionally varied with a ratio of ATP:dATP:dGTP:dTTP being 170:4:1:3.

Effects of Variations in Concentration of Individual dNTPs

In an attempt to understand differential changes in product formation resulting from simultaneous variations in dNTP concentration, a series of four-substrate assays was carried out under quasi-physiological conditions with only one allosteric effector being varied at a time. Apparently, dATP as well as dGTP and dTTP had differential effects on reduction of all four substrates in a concentration-dependence manner.

i) Effect of dATP

As shown in Figure 4.16, at low concentration (≤ 20 μM), dATP activated pyrimidine nucleotide reduction, but inhibited purine reduction. As dATP concentration increased, GDP reduction was inhibited, whereas ADP reduction was activated. Reduction of pyrimidine nucleotides, nonetheless, showed no significant changes.

ii) Effect of dGTP

Variations in dGTP concentration resulted in a decrease of CDP reduction and an increase of purine reduction. Reduction of UDP remained essentially unchanged (Figure 4.17).

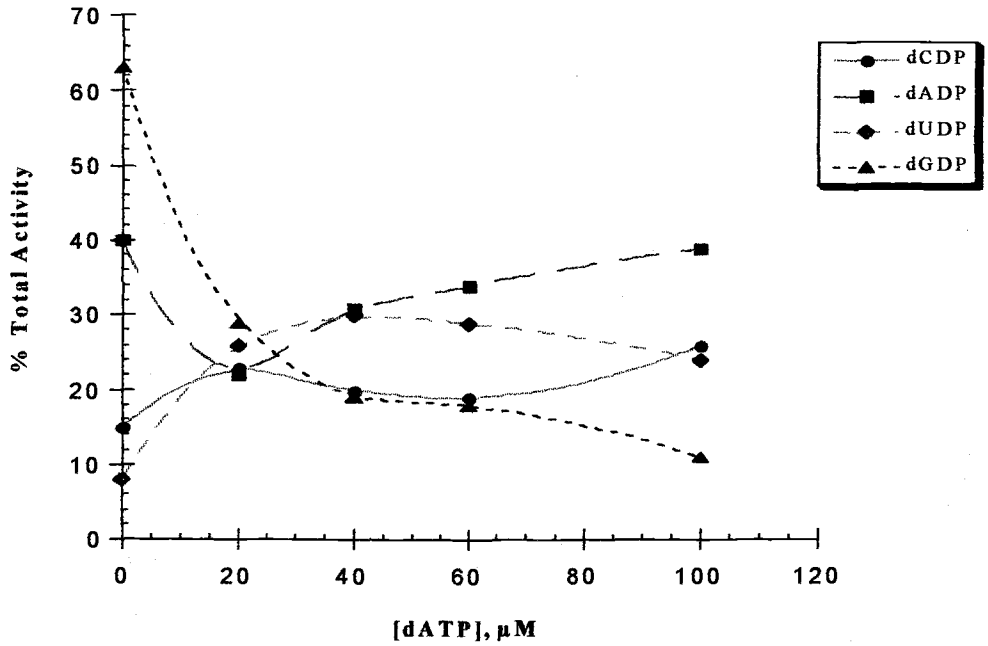


Figure 4.16 Effect of varied concentrations of dATP on mouse rNDP reductase activities. The enzyme was analyzed in the presence of quasi-physiological concentrations of rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP) and allosteric modifiers (2.5 mM ATP, 60 μM dATP, and 15 μM dGTP). The amount of dATP was varied as indicated.

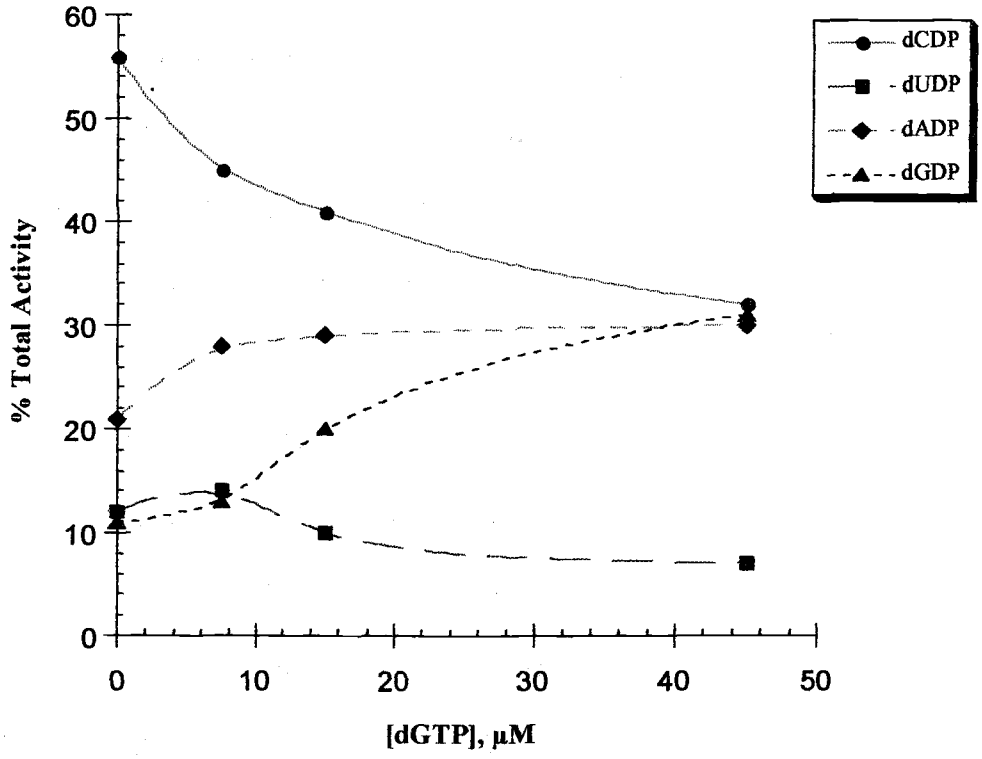


Figure 4.17 Effect of varied concentrations of dGTP on mouse rNDP reductase activities. The enzyme was analyzed in the presence of quasi-physiological concentrations of rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP) and allosteric modifiers (2.5 mM ATP, 60 μM dATP, and 15 μM dGTP). The amount of dGTP was varied as indicated.

iii) Effect of dTTP

Figure 4.18 demonstrates the effect of dTTP on enzyme specificity. GDP reduction slightly increased with increasing concentrations of dTTP, whereas reduction of ADP showed a slight decrease. Reduction of UDP was significantly inhibited. Interestingly, CDP reduction was activated at low dTTP concentrations, and slightly inhibited after dTTP concentration was higher than its physiological concentration (50 μM).

The activation of CDP reduction by dTTP was quite surprising, given the consistent identification of dTTP as an inhibitor of CDP reduction in single-substrate assays (Eriksson *et* al., 1979; Thelander *et* al., 1980; Reichard, 1993; Reichard *et* al., 2000). Two additional experiments were designed to eliminate other possible explanations by simplifying conditions under which the effect of dTTP was examined. In the first experiment, dTTP concentration was varied in the presence of CDP as a single substrate and all allosteric effectors at their physiological concentrations to determine whether the increase of CDP reduction was due to effects of other substrates (Figure 4.19). This could be a reasonable explanation since ADP was previously shown to inhibit purine nucleotide reduction, but to activate reduction of CDP. The second experiment involved varying dTTP concentration in the presence of all four substrates with only dTTP and ATP as modifiers in order to determine whether the effects seen were solely due to dTTP, not other modifiers (Figure 4.20). Results from both experiments

showed the same effect of dTTP on CDP reduction. As dTTP concentration was increased, reduction of CDP was inhibited. When only dTTP and ATP were present as allosteric modifiers with all four substrates, pyrimidine nucleotide reduction was inhibited, whereas purine reduction was activated, consistent with results from a single-substrate assay and the four-substrate assay carried out with a constant dTTP concentration.

iv) Effect of dCTP

The effect of dCTP was examined in reaction mixtures containing all four substrates with only dCTP and ATP as allosteric modifiers. Variations in dCTP concentration under this condition essentially had no effect upon four activities of the enzyme (Figure 4.21). However, when other dNTP regulators were included (Figure 4.22), differential reduction of the four substrates became apparent, suggesting a feasible influence of other dNTPs on binding of dCTP to the large subunit of RNR which in turn, affected reduction of four substrates differentially.

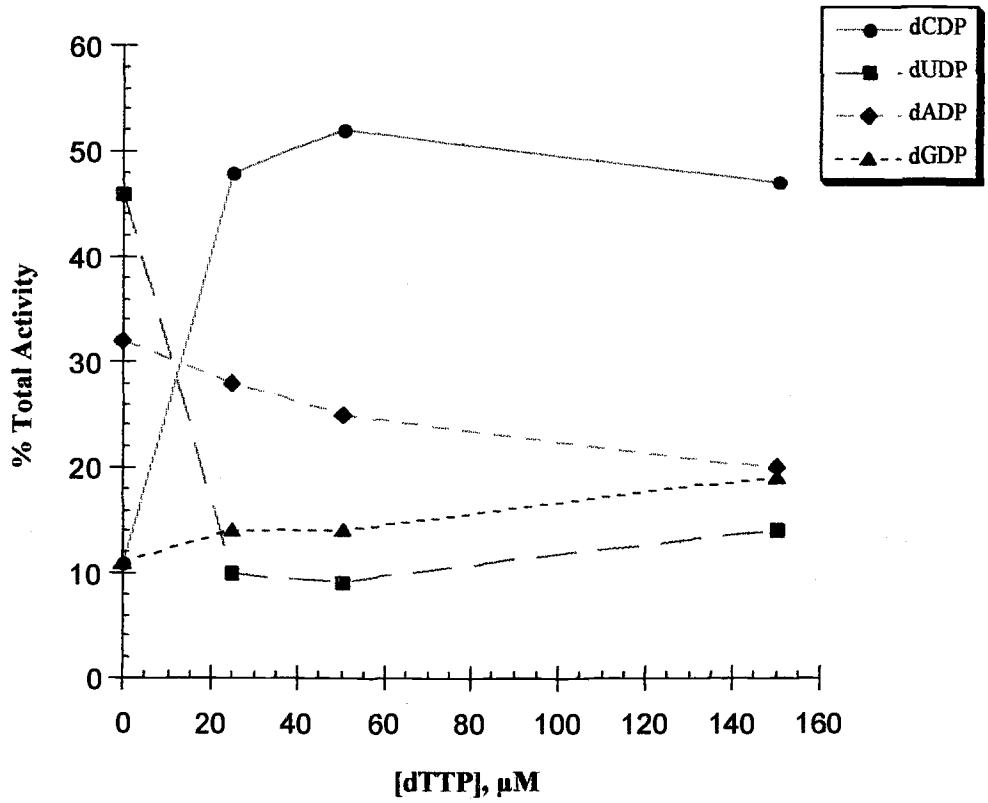


Figure 4.18 Effect of varied concentrations of dTTP on mouse rNDP reductase activities. The enzyme was analyzed in the presence of quasi-physiological concentrations of rNDP substrates (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP) and allosteric modifiers (2.5 mM ATP, 60 μM dATP, and 15 μM dGTP). The amount of dTTP was varied as indicated.

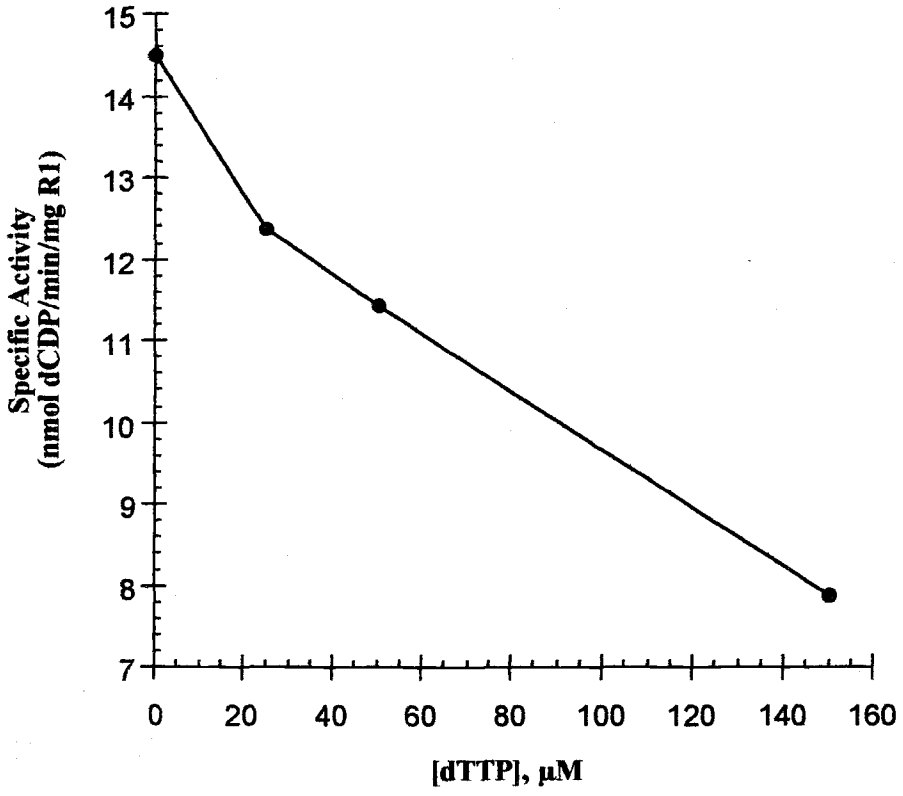


Figure 4.19 Effect of varied concentrations of dTTP on CDP reductase activity of mouse ribonucleotide reductase. In this experiment, CDP was the only substrate present at a final concentration of 70 μM. The allosteric modifiers, on the hand, were added at their approximate physiological concentrations (2.5 mM ATP, 60 μM dATP, and 15 μM dGTP) with indicated amounts of dTTP.

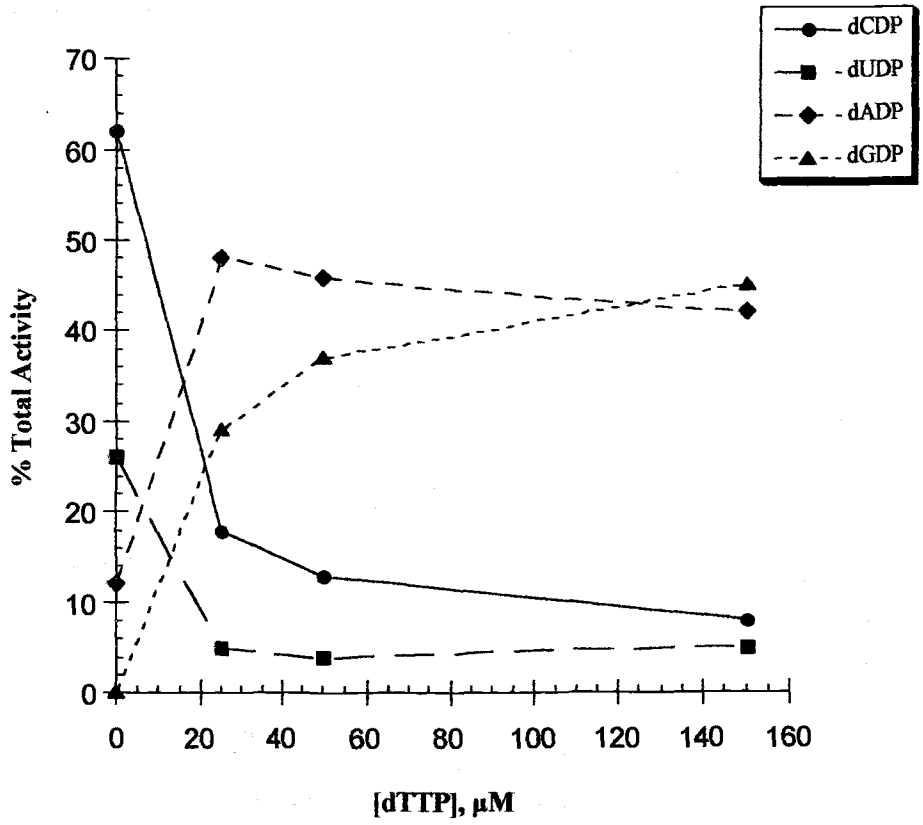


Figure 4.20 Effect of varied concentrations of dTTP on mouse rNDP reductase activities in the presence of only dTTP and ATP. The reaction mixtures contained all four substrates at their estimated *in vivo* concentrations (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP). However, only varied concentrations of dTTP (as indicated) and 2.5 mM ATP were present.

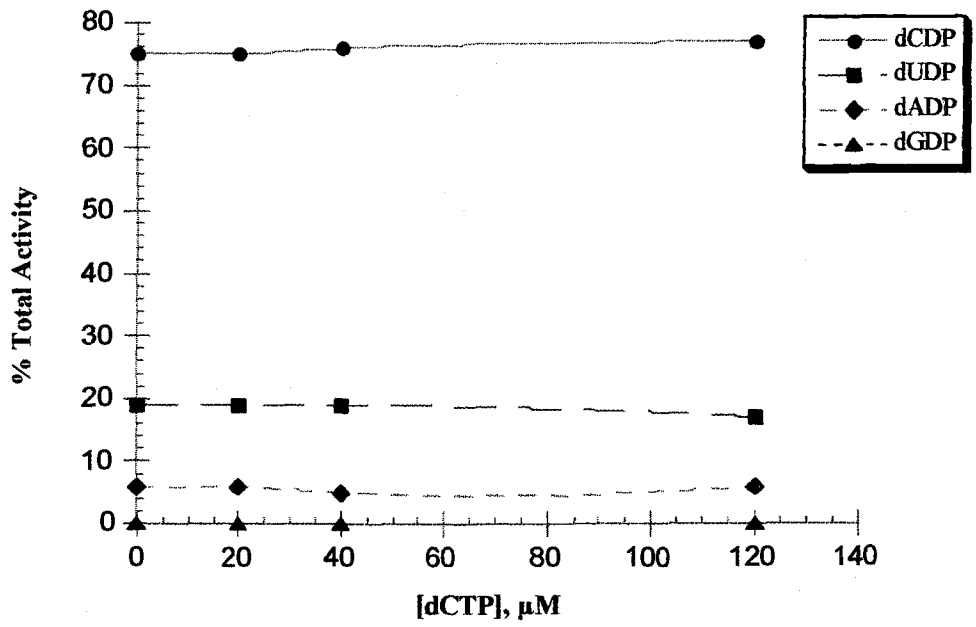


Figure 4.21 Effect of varied concentrations of dCTP on mouse rNDP reductase activities with only dCTP and ATP as allosteric modifiers. The four activities were assayed in the presence of all four substrates at their "*in vivo*" concentrations (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP). However, only dCTP and 2.5 mM ATP were present as allosteric regulators. The amount of dCTP was varied as shown.

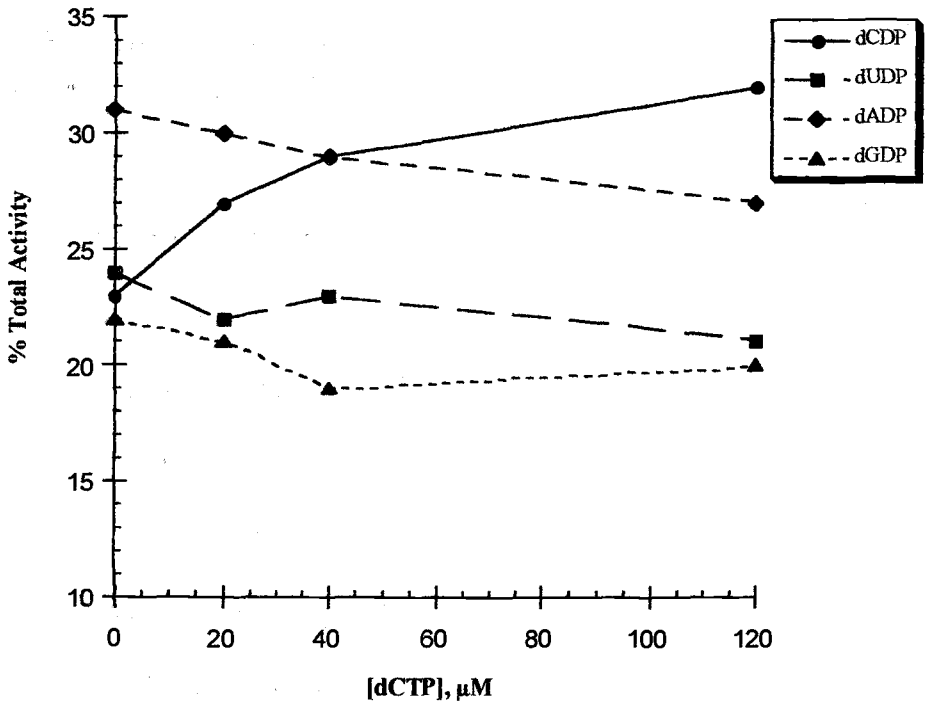


Figure 4.22 Effect of varied concentrations of dCTP on mouse rNDP reductase activities in the presence of all four effectors. The assay conditions were the same as those in Figure 4.21 except that allosteric effectors were included at their estimated *in vivo* concentrations (2.5 mM ATP, 60 μM dATP, 15 μM dGTP and 50 μM dTTP). The concentration of dCTP was varied as indicated.

Chapter 5

Influence of Substrate Binding upon Mammalian Ribonucleoside Diphosphate Reductase Specificity

It has been shown, in Chapter 4, that binding of individual allosteric modifiers to the large subunit of mouse ribonucleotide reductase (RNR) differentially affects reduction of all four substrates. Each dNTP has an important role in facilitating or inhibiting reduction of certain rNDPs, depending on its concentration present in the reaction mixtures, thus presumably also inside the cell. This indicates one way to regulate the overall activity and specificity of RNRs in order to achieve a proportional production of DNA building blocks. This chapter, however, will focus on effects of variations in rNDP substrate concentration upon enzyme specificity. It has been reported for bacteriophage T4 (Hendricks and Mathews, 1997) and vaccinia virus (Hendricks and Mathews, 1998) RNRs that substrate concentrations are equally crucial as allosteric modifier concentrations in maintaining product formation rates proportional to the base composition of the genome. Furthermore, by using vaccinia virus RNR, it has been revealed that ADP reduction is specifically inhibited by ADP itself, suggesting a feasible regulatory role of rNDP substrates (at least of ADP) (Hendricks and Mathews, 1998).
The question of whether or not this is the case for mammalian RNR, nonetheless,

remains to be determined. In addition, it is interesting to learn how variations in concentration of rNDP substrates influence enzyme specificity, as well as whether their effects are involved in cellular regulation.

Changes of Mouse Ribonucleotide Reductase Product Profiles Due to Various Assay Conditions

Variations in rNDP substrate and dNTP allosteric modifier concentrations resulted in different product profiles. As shown in Table 5.1, when dNTPs were omitted from the reaction mixtures, production of all four substrates appeared highly imbalanced. With either equimolar (0.15 mM each) or estimated *in vivo* (850 μM ADP, 70 μM CDP, 160 μM GDP and 160 μM UDP) substrates, CDP was the most favored substrate, resulting in nearly 80% dCDP of total product formed (almost 4-fold greater than the expected value). Additionally, relative rates of formation of dADP, dGDP and dUDP were vastly lower than reported values of corresponding nucleotides in the mouse genome. It was evident that the presence of dNTPs at their *in vivo* concentrations was pivotal for maintaining biologically balanced deoxyribonucleotide formation. In fact, rates of dNDP product formation were the closest to rates of corresponding nucleotide utilization when both substrates and allosteric effectors were added to the reaction mixtures at their quasi-physiological concentrations (60 μM dATP, 15 μM dGTP, and 50 μM dTTP). Since there are some noticeable changes in product profiles when the

enzyme is exposed to equimolar substrates and to estimated *in vivo* substrate concentrations in the presence of *in vivo* dNTPs, a possibility that substrates themselves directly are involved in controlling dNDP product formation cannot be ruled out. Besides, because the four rNDP substrates compete for binding to the same catalytic site, it would be surprising if they did not exert control.

Table 5.1 Effects of allosteric modifier and substrate concentrations on mammalian ribonucleotide reductase specificity. ATP was present in all reaction mixtures at 2.5 mM since it was strictly required for enzyme activity. Where indicated, dNTPs were present at their estimated physiological concentrations (60 μM, 15 μM, and 50 μM for dATP, dGTP and dTTP, respectively). Equimolar rNDPs refers to 0.15 mM each of ADP, CDP, GDP and UDP, whereas, *in vivo* rNDPs refers to 850 μM, 70 μM, 160 μM and 160 μM of ADP, CDP, GDP and UDP, respectively.

Assay conditions	Percentage of total product formed			
	dCDP	dUDP	dADP	dGDP
Equimolar rNDPs, no dNTPs	78	18	4	<1
Equimolar rNDPs, *in vivo* dNTPs	28	26	33	14
In vivo rNDPs, no dNTPs	78	19	3	<1
In vivo rNDPs, *in vivo* dNTPs	34	25	27	15
Percentage of total nucleotides in mouse genome	21	29*	29	21

* Present in mouse DNA as dTMP.

Proportional Variations in Substrate Concentration

To investigate effects of rNDP substrates upon enzyme specificity, the four-substrate assay was carried out under approximate physiological conditions. All allosteric modifiers were present at their estimated *in vivo* concentrations, whereas rNDP substrates were bioproportionally varied; the ratio of ADP: CDP: GDP: UDP was essentially held constant at 12:1:2:2, respectively, in accord with ratios of these four rNDPs in animal cells (Traut, 1994). Interestingly, a proportional increment of substrate concentrations revealed inhibitory effects of substrates on reduction of purine nucleotides—ADP and GDP. Reduction of pyrimidine nucleotides was affected differently. CDP reduction was activated while UDP reduction was unchanged (Figure 5.1).

Effects of Variations in Individual Substrate Concentrations on Mouse RNR Specificity

To examine which substrate(s) contributed to differential effects seen in the previous experiment, the concentrations of ADP, CDP, GDP and UDP were individually varied. Substrate concentrations used ranged from 850-1800 μM, 70-630 μM, 160-1440 μM, 160-1440 μM for ADP, CDP, GDP and UDP, respectively. While each substrate was varied as indicated, the other three rNDPs and all allosteric effectors were held constant at their estimated physiological concentrations. As illustrated in Figure 5.2, increasing concentrations of CDP, GDP

and UDP resulted in an activation of reduction of the corresponding varied substrates (either CDP, GDP or UDP) and an inhibition of reduction of other substrates present at their fixed concentrations, suggesting a substrate competition phenomenon for binding at the catalytic site. For instance, when GDP concentrations were varied from 160 μM to over 1.4 mM, formation of dGDP increased, whereas formation of dADP, dCDP, and dGDP decreased (Panel A, Figure 5.2). This could be due to the fact that the chance of higher concentration of GDP competing with other substrates and binding to the catalytic site was greater than that of other substrates. Therefore, more GDP molecules were bound and converted to a corresponding product as a result of winning the competition. Similar effects were achieved with increasing concentrations of CDP and UDP (Panel B and C, respectively, Figure 5.2).

Effects of ADP upon Mouse RNR Specificity

An interesting result arose when the concentration of ADP was varied, as those of others were held constant at their approximate *in vivo* concentrations. As shown in Figure 5.3, ADP at high concentrations inhibited its own reduction and that of GDP, but activated CDP reduction, indicating a more complex influence of ADP on enzyme specificity than a substrate competition. If effects seen here resulted from a competition among rNDP substrates, dADP formation would be elevated, while formation of dCDP, dGDP and dUDP would be inhibited. In fact,

the data resembled those obtained in a previous experiment in which substrate concentrations were proportionately varied (Figure 5.1). At least partially, the inhibitory effect of ADP was in agreement with what was observed with RNR encoded by vaccinia virus (Hendricks and Mathews, 1998). In addition, differential effects of ADP were further confirmed with two more experiments. First, the four-substrate assay was carried out in the presence of varied ADP with other three substrates held constant at their estimated *in vivo* concentrations. Only ATP was present as an activator for the enzyme to rule out the possibility that other dNTP modifiers may have exerted their effects on the enzyme specificity. As shown in Figure 5.4, a consistent inhibitory effect of high concentrations of ADP on ADP reduction was obtained. CDP reduction was activated as expected. Besides, an additional inhibitory effect on UDP reduction was more pronounced under this assay condition. By contrast, GDP reductase activity was undetectable because dTTP was omitted. The second experiment was performed the same way as the first, except that dTTP at 50 μM (*in vivo* concentration) was included. This was to confirm the inhibitory effect of ADP on GDP reduction since without dTTP, dGDP formation was not measurable. It turned out that the influence of ADP on enzyme specificity remained consistent (Figure 5.5). As ADP concentrations increased, reduction of purine nucleotdes was inhibited, whereas CDP reduction was activated. This experiment apparently showed that ADP, not dNTP modifiers, was probably responsible for differential effects on the specificity of mouse RNR. Otherwise, due to the allosteric regulation by dTTP, one would expect a primary

increase of GDP reduction as well as ADP reduction, accompanied by a decrease in reduction of pyrimidine nucleotides. Hence, these data have suggested that ADP may have a regulatory role towards eukaryotic ribonucleoside diphosphate reductases.

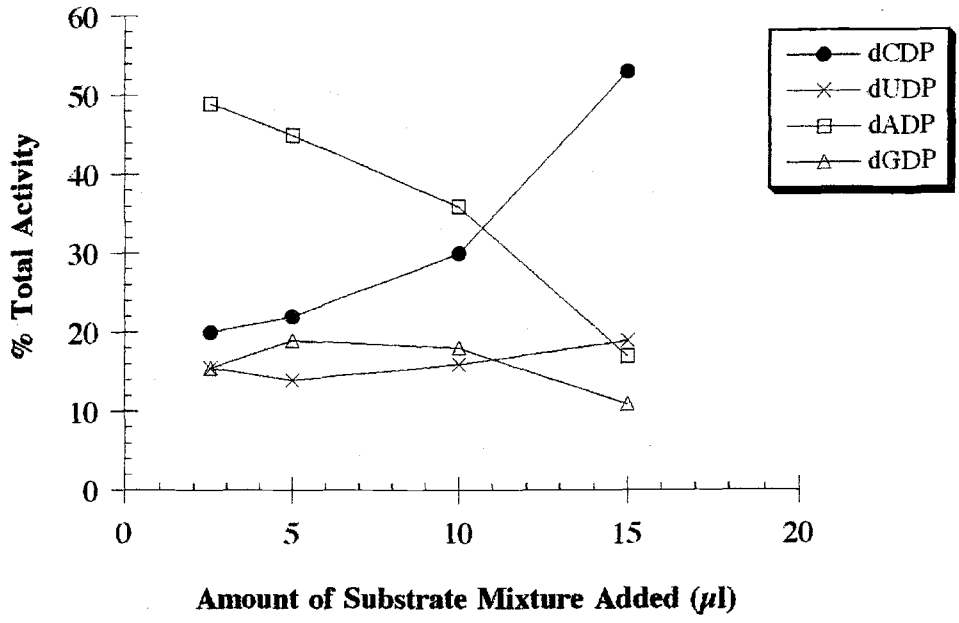


Figure 5.1 Effects of proportional variations in substrate concentrations upon the four activities of mouse ribonucleotide reductase. The reaction mixture contained a combination of all four rNDP substrates at a ratio of ADP:CDP:GDP:UDP being 12:1:2:2, and allosteric modifiers at their estimated *in vivo* concentrations (2.5 mM ATP, 60 μM dATP, 15 μM dGTP, and 50 μM dTTP).

Figure 5.2 Effects of variations in concentrations of individual substrates upon the four activities of mouse ribonucleotide reductase. In all three experiments, concentrations of GDP, CDP or UDP were varied (one rNDP at a time), whereas concentrations of the other three rNDP substrates and all allosteric effectors were held constant at their quasi-physiological concentrations. **A, effect of GDP.** Concentrations of GDP were varied from 160-1440 μM. **B, effect of CDP.** Concentrations of CDP were varied from 70-630 μM. **C, effect of UDP.** Concentrations of UDP were varied from 160-1440 μM.

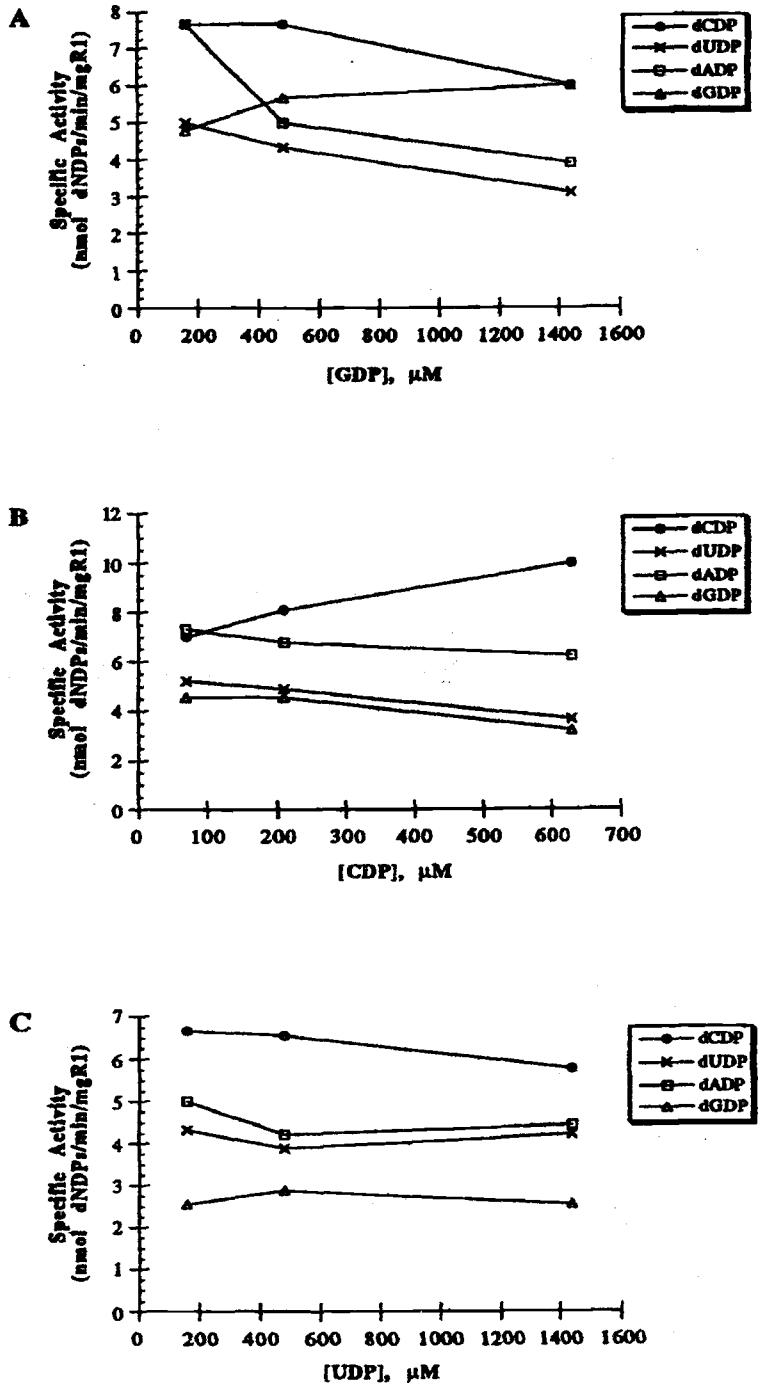

A
Specific Activity
(nmol dNDPs/min/mgR1)
dCDP
dUDP
dADP
dGDP
[GDP], µM
B
Specific Activity
(nmol dNDPs/min/mgR1)
dCDP
dUDP
dADP
dGDP
[CDP], µM
C
Specific Activity
(nmol dNDPs/min/mgR1)
dCDP
dUDP
dADP
dGDP
[UDP], µM

Figure 5.2

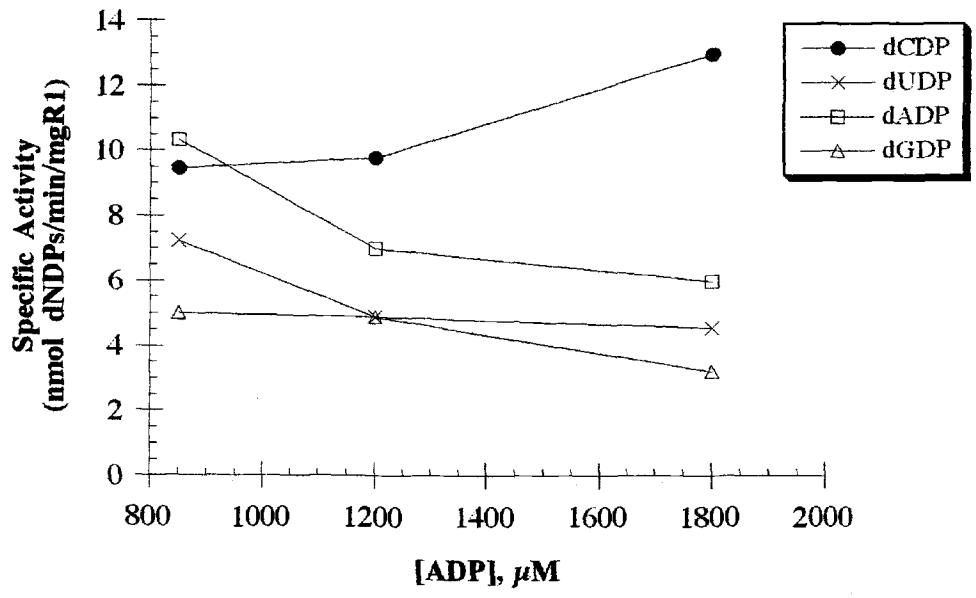


Figure 5.3 Effects of variations in ADP concentration on upon the four activities of mouse ribonucleotide reductase. The four-substrate assay was performed in the presence of varied concentrations of ADP as indicated (from 850-1800 μM). The other three rNDP substrates (CDP, GDP and UDP) and all allosteric modifiers were held constant at their estimated *in vivo* concentrations.

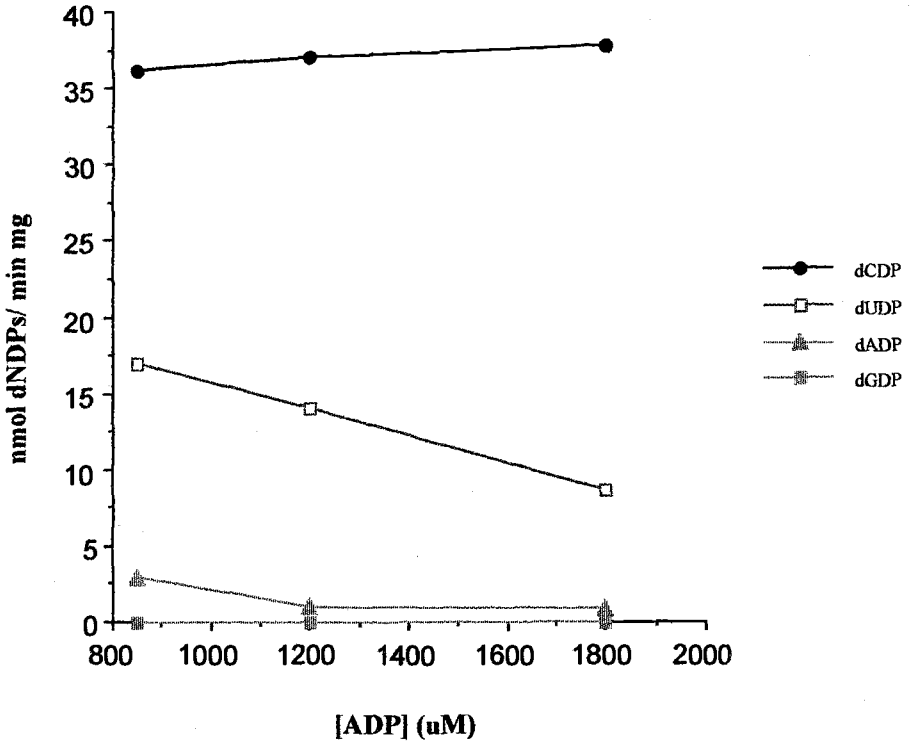


Figure 5.4 Effects of variations in ADP concentration on upon the four activities of mouse ribonucleotide reductase with ATP as a sole activator. The four-substrate assay was carried out in the presence of 1 μM mR1, 2 μM mR2, 2.5 mM ATP (the only modifier present), and rNDP substrates at their estimated *in vivo* concentrations. Concentrations of ADP were varied as indicated.

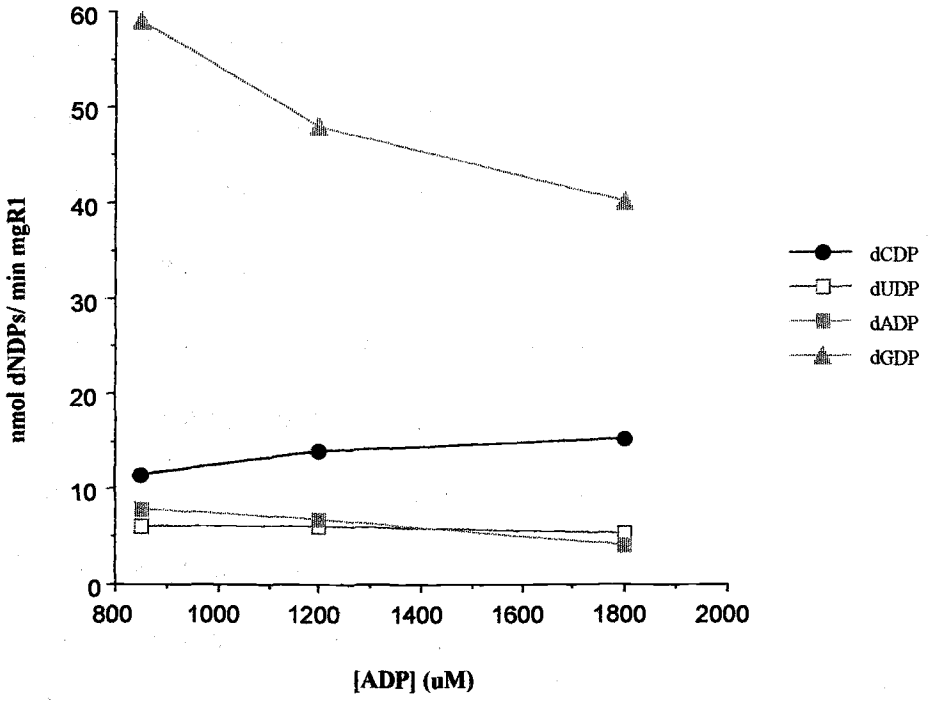


Figure 5.5 Effects of variations in ADP concentration on upon the four activities of mouse ribonucleotide reductase in the presence of ATP and dTTP. In this experiment, the assay conditions were the same as in Figure 5.4 except that 50 μM of dTTP was added to activate GDP reduction.

Biological Significance of ADP Inhibition

A potential biological significance of autoinhibition of ADP may involve the link between DNA replication and energy metabolism inside the cell. At a low energy state (high ADP/ATP ratio), cells may have to reduce the rate of ADP reduction in order to avoid accumulation of dATP which can, in turn, inhibit ribonucleotide reductase, eventually resulting in DNA replication discontinuity or apoptosis (Reichard, 1988). If this is the case, the dATP level in cells that exhibit a high ADP/ATP ratio should be specifically lowered compared to other dNTP pools. To test this hypothesis, V79 hamster lung cells were cultured as described in Chapter 3, and treated with either 2, 4-dinitrophenol (DNP) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) in an attempt to increase the ADP/ATP ratio by partial uncoupling of oxidative phosphorylation. Tables 5.2a and 5.2b show amounts of ribonucleotides (measured by HPLC analysis of total nucleotides in cell extracts subtracted by the amount of deoxyribonucleotides) and deoxyribonucleotides (measured by boronate chromatography followed by HPLC analysis), in untreated and DNP-treated cells obtained from two independent experiments. Evidently, cell extracts contained deoxyribonucleotides at less than 10% of the values for total nucleotides. In fact, many deoxyribonucleotide counterparts were present at such low levels that they were not detectable by such a sensitive method as HPLC. The ADP/ATP ratio was then calculated from values in Tables 5.2a and 5.2b. As depicted in Table 5.3, there were no significant changes in

the ADP/ATP ratio when V79 cells were treated with DNP. Two independent experiments resulted in similar ADP/ATP values ranging from 0.10 to 0.20.

Table 5.2a Amounts of nucleotides in V79 cell extracts (Experiment 1). After cells were treated with varied concentrations of DNP for 2 hours, cells were examined under a microscope to observe cytotoxicity (No morphological changes were observed.). Cell extracts were then prepared by using a standard protocol described in Chapter 3. Since DNP needed to be dissolved in 95% ethanol, controls that were treated with corresponding percentage of ethanol were also included. Concentrations of DNP at 0.25 mM, 0.5 mM, and 1.0 mM contained 0.4%, 0.9%, and 1.7% ethanol (EtOH), respectively. For this particular experiment, cell extracts were prepared from one plate of V79 cells with a cell density of 1.71×10^5cells/ml/treatment condition. Amounts of ribonucleotides are presented in nanomoles per 10^6 cells, whereas deoxyribonucleotides are presented as percentage of total nucleotides.

rNT	U		.25mM DNP		0.5mM DNP		1 mM DNP		0.4% EtOH		0.9% EtOH		1.7% EtOH	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
CDP	12	1	10	1	10	1	10	N	8	N	10	N	11	N
UDP	4	2	3	5	3	3	4	2	2	4	3	3	5	3
ADP	15	4	12	5	14	4	17	4	9	8	11	6	17	3
GDP	5	1	4	2	5	1	5	N	3	N	4	N	5	N
CTP	6	5	7	1	6	1	7	1	6	5	7	3	7	4
UTP	48	1	56	2	52	1	50	1	60	2	63	1	57	1
ATP	80	N	104	N	91	N	83	N	99	N	101	N	86	N
GTP	23	N	27	1	25	N	25	N	26	N	28	N	23	N

Note: rNT = Ribonucleotides; U = Untreated cells; N = Non-detectable(<100 pmol)
A = Nanomoles of ribonucleotides per 10^6 cells; B = Percentage of corresponding deoxyribonucleotides

Table 5.2b Amounts of nucleotides in V79 cell extracts (Experiment 2).
Cells were treated the same way as those in Table 5.2a. However, cells extracts were prepared from two plates of cells with the cell density of 1.74×10^5 cells/ml/treatment condition.

rNT	U		.25mM DNP		0.5mM DNP		1 mM DNP		0.4% EtOH		0.9% EtOH		1.7% EtOH	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
CDP	6	2	5	3	7	1	7	3	6	2	6	1	7	2
UDP	3	3	4	3	4	3	5	4	4	4	3	6	5	2
ADP	13	2	15	1	16	2	15	1	14	2	10	3	16	2
GDP	3	N	3	N	4	N	4	1	3	N	2	N	3	1
CTP	4	9	3	4	4	2	4	5	4	9	4	9	5	9
UTP	13	5	10	4	13	3	13	5	15	4	15	4	16	5
ATP	109	N	88	N	107	N	97	N	117	N	117	N	117	1
GTP	6	1	5	1	7	N	7	1	6	2	7	1	6	N

Note: rNT = Ribonucleotides; U = Untreated cells; N = Non-detectable(<100pmol)
A = Nanomoles of ribonucleotides per 10^6 cells; B = Percentage of corresponding deoxyribonucleotides

Table 5.3 Effects of 2, 4-dinitrophenol (DNP) upon ADP/ATP ratios of V79 hamster lung cells under different treatment conditions. Amounts of ADP and ATP in untreated and DNP-treated or EtOH-treated cell extracts illustrated in Table 5.2a and 5.2b were used to calculate ADP/ATP ratios in order to compare changes in cells' energy states induced by various treatment conditions.

Treatment conditions	ADP/ATP ratio	
	Experiment 1	Experiment 2
Untreated cells	0.20	0.13
0.25 mM DNP	0.11	0.17
0.50 mM DNP	0.15	0.14
1.00 mM DNP	0.20	0.17
0.4% EtOH	0.10	0.13
0.9% EtOH	0.11	0.10
1.7% EtOH	0.20	0.14

However, when V79 hamster lung cells were treated with FCCP at concentrations ranging from 0.5 to 10 mM, ATP levels were remarkably decreased compared to the untreated control, suggesting a successful block of ATP synthesis caused by the uncoupler FCCP. In addition to ATP blockage, synthesis of two other nucleoside triphosphate species—GTP and UTP, was also slightly inhibited. Yet changes in nucleotide levels, as mentioned, were consistent in two independent experiments (Tables 5.4a and 5.4b). The ADP/ATP ratio for each treatment condition was calculated and presented in Table 5.5. The first experiment yielded ADP/ATP ratio changes ranging from 1.3- to 2.8-fold relative to untreated control,

whereas the second yielded from 1.7- to 4.2-fold increases. Although changes in the ADP/ATP ratio varied with treatment conditions in an FCCP concentration-independent manner in both experiments, these changes were significant enough to carry out further investigation to test the original hypothesis.

Table 5.4a Amounts of ribonucleotides in untreated and FCCP-treated V79 hamster lung cells measured by HPLC (Experiment 1). After cells were incubated with varied concentrations of FCCP for two hours, cell extracts were prepared using a standard protocol described in Chapter 3. Each extract was then applied to HPLC for nucleotide measurement (cell density = 3.50×10^5 cells/ml).

Ribonucleotides	Amounts of nucleotides under various treatment conditions (nmol/10^6 cells)				
	Untreated Control	0.5 mM FCCP	1 mM FCCP	5 mM FCCP	10 mM FCCP
CDP	4.04	3.97	3.01	3.28	2.52
UDP	2.07	2.47	1.38	2.25	1.68
ADP	6.01	12.78	6.29	9.78	5.10
GDP	1.35	3.50	1.88	2.92	2.69
CTP	3.68	2.28	2.54	2.61	2.52
UTP	13.65	6.30	7.52	5.36	5.62
ATP	39.62	30.62	33.19	22.97	16.29
GTP	12.20	12.25	14.24	9.43	6.60

Table 5.4b Amounts of ribonucleotides in untreated and FCCP-treated V79 hamster lung cells measured by HPLC (Experiment 2). V79 cells were treated with same concentrations of FCCP as in Experiment 1. Cell extracts were prepared and analyzed by the same procedure, but with slightly lower cell density (2.72×10^5 cells/ml).

Ribonucleotides	Amounts of nucleotides under various treatment conditions (nmol/10^6 cells)				
	Untreated Control	0.5 mM FCCP	1 mM FCCP	5 mM FCCP	10 mM FCCP
CDP	3.21	3.53	3.33	3.42	2.38
UDP	0.86	1.20	1.05	1.64	0.90
ADP	2.05	3.02	2.57	3.23	1.13
GDP	0.78	1.43	1.27	1.94	1.13
CTP	3.51	1.87	1.89	1.96	3.10
UTP	9.40	4.52	4.52	4.13	5.73
ATP	35.56	18.09	18.23	12.70	11.76
GTP	12.76	9.28	9.65	7.84	8.39

Table 5.5 Effects of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) upon ADP/ATP ratios of V79 hamster lung cells under different treatment conditions. Calculation was performed using numbers in Tables 5.4a and 5.4b.

mM FCCP	Experiment 1		Experiment 2	
	ADP/ATP ratios	ADP/ATP changes (fold)	ADP/ATP ratios	ADP/ATP changes (fold)
0.0	0.15	-	0.06	-
0.5	0.41	2.7	0.17	2.8
1.0	0.19	1.3	0.14	2.3
5.0	0.42	2.8	0.25	4.2
10.0	0.31	2.1	0.10	1.7

To test our hypothesis that the inhibitory effect of ADP upon its own reduction is to prevent dATP accumulation inside the cell, the amounts of dNTPs in untreated and FCCP-treated V79 hamster lung cells were determined by using the dNTP pool enzymatic assay. If the hypothesis is correct, dATP pool should be specifically lowered while other pools are unaffected by the decrease of ADP/ATP ratios. Table 5.6 illustrates dNTP pool measurements of V79 hamster lung cells treated with 0, 0.5, 1, 5, and 10 mM FCCP in two independent experiments. Interestingly, all dNTP pools in FCCP-treated cells slightly decreased compared to those in the untreated control in an FCCP concentration-independent manner. However, changes in dNTP pools did not correspond to ADP/ATP ratio

modification caused by FCCP (Table 5.5). Hence, even though the amount of dATP did decline, it was not significant enough to draw a conclusion that the inhibitory effect of ADP represented a link between the energy metabolism and DNA replication.

Table 5.6 Amounts of dNTPs measured by the dNTP pool enzymatic assay. Two sets of cell extracts prepared from V79 hamster lung cells treated with varied concentrations of FCCP and the untreated control were subjected to the dNTP pool enzymatic assay as described in Chapter 3. The two experiments carried out at different periods of time resulted in different pool values due to different energy states (Table 5.5). However, the tendency of changes in dNTP pools remained similar.

mM FCCP	Amounts of dNTPs under various treatment conditions (pmol/10^6 cells)							
	dATP		dCTP		dGTP		dTTP	
	1	2	1	2	1	2	1	2
0.0	546.0	618.0	372.7	565.2	108.3	170.1	465.8	752.5
0.5	493.2	317.7	298.2	252.4	161.3	143.8	453.8	579.0
1.0	453.7	411.7	295.7	263.7	138.0	91.0	447.2	564.0
5.0	420.5	393.1	322.1	307.7	63.3	135.0	456.9	482.7
10.0	417.3	313.7	366.3	419.7	133.4	207.2	452.3	583.9

Note: 1 = Experiment 1; 2 = Experiment 2

Effects of rNDP Substrates on Binding of Allosteric Modifiers to the Large Subunit of Mouse Ribonucleotide Reductase (mR1)

As shown in Figure 5.3, high concentrations of ADP inhibit purine nucleotide reduction, but activate CDP (and possibly UDP) reduction, suggesting a tentative regulatory effect of ADP on the enzyme specificity. In fact, this must be more complicated than simple competition between ADP and other rNDP substrates at the catalytic site because increasing concentrations of ADP lead to a decrease in dADP formation and an increase in dCDP, instead of the opposite. Therefore, it was interesting to investigate the mechanism by which ADP affects the product profile of mouse RNR or its enzyme specificity. ADP, perhaps, can also bind to the activity site and/or the specificity site at high concentrations due to its structural resemblance to ATP. Or even the possibility that ADP has a novel unknown binding site on the large subunit of the enzyme cannot be ruled out.

Binding of dGTP, dTTP and dATP to Mouse R1 Protein in the Absence of rNDP Substrates

In order to understand the binding of ADP to mouse R1, nucleotide binding experiments were performed using the ultrafiltration assay described in detail in Chapter 3. Specifically, it was of interest to examine whether high concentrations of ADP influence binding of dNTP modifiers to the specificity site of the enzyme, implying the ability of ADP to displace dNTPs and thus to bind at the particular

site. As illustrated in Figure 5.6, the binding of each dNTP to mouse R1 was first determined in the absence of ADP or any other substrates. Hyperbolic curves were obtained for binding of both dGTP (Figure 5.6 A) and dTTP (Figure 5.6 B) with the Y-interception at 1 as expected for a single binding site per R1 monomer. Linear regression analysis resulted in binding constants (K_d) of 4.4 ± 0.38 μM and 1.8 ± 0.30 μM for dGTP and dTTP, respectively. The binding of dATP was analyzed at two different concentrations of mR1 protein due to the fact that dATP could bind at two different sites—the activity (low affinity) site and the specificity (high affinity) site. By using a Scatchard plot as an effective way to present the data, two straight lines were obtained indicating binding constants of 54.3 ± 4.0 μM and 12.6 ± 2.3 μM for the low and high affinity sites, respectively (Figure 5.6 C).

Figure 5.6 Determination of binding constants of allosteric effectors to mouse R1 protein. A, dGTP binding. Mouse R1protein was present at 6 μM. **B, dTTP binding.** Mouse R1protein was present at 2 μM. For A and B, K_d values were determined by nonlinear least squares fit by Kaleidagraph 3.5. **C. dATP binding.** Data were collected at two different R1 concentrations as indicated, and analyzed by linear regression on a Scatchard plot.

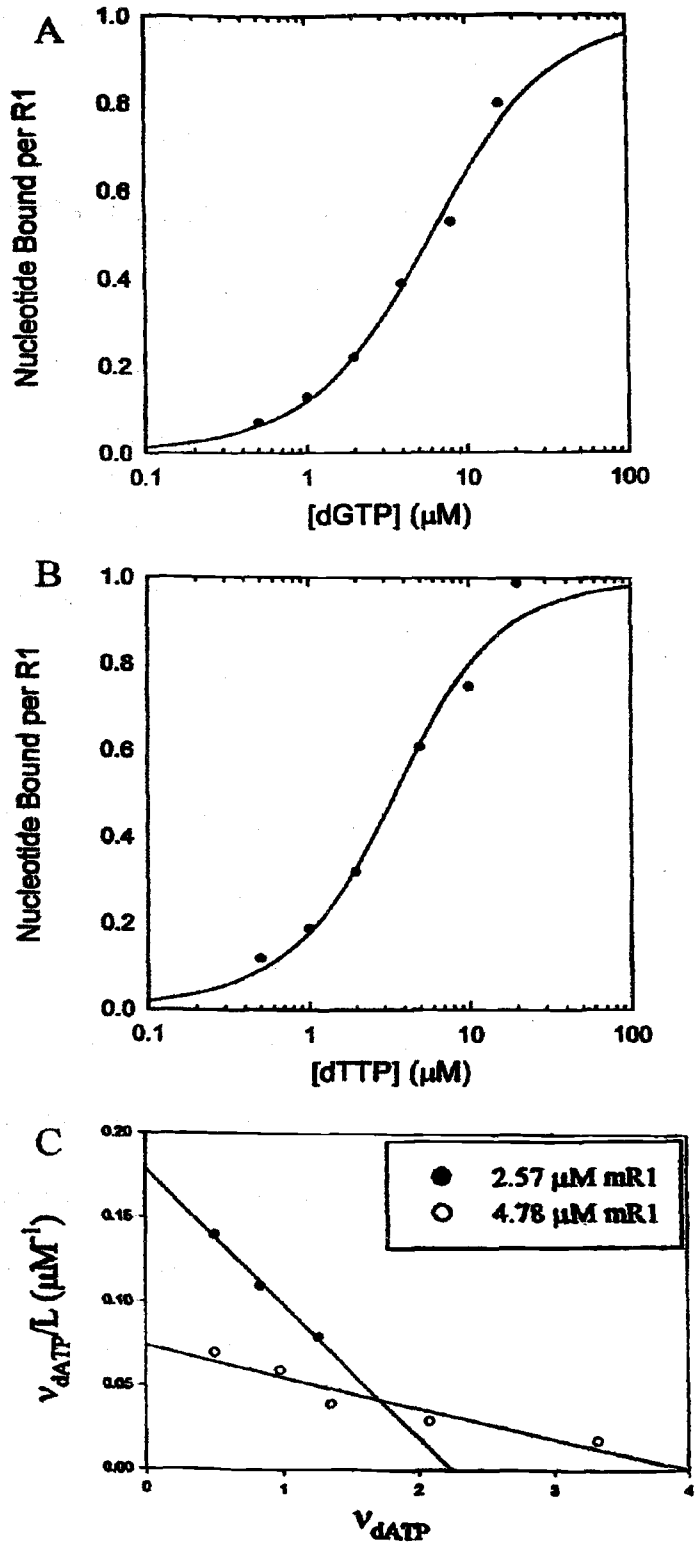

A
Nucleotide Bound per R1
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
[dGTP] (μM)
B
Nucleotide Bound per R1
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
[dTTP] (μM)
C
v_{dATP}/L (μM^{-1})
2.57 μM mR1
4.78 μM mR1
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
v_{dATP}

Figure 5.6

The Direct Effect of ADP on Binding of dATP to the Mouse R1

To examine whether ADP could bind to additional sites such as the activity site and the specificity site or both, ADP was included in the ultrafiltration assay to compete with dATP in binding to those two sites on mR1 protein. The concentration of dATP was held constant at 1.5 μM, its K_d as reported by Reichard *et al.* (2000), whereas ADP concentrations were varied from 0 to 1800 μM. Changes of dATP binding to mR1 were then monitored as concentrations of ADP increased. As shown in Figure 5.7, ADP at concentrations as high as 1800 μM had no effect upon dATP binding (open circles). Since ADP could bind primarily to the catalytic site, CDP was included to minimize binding of ADP to this particular site. As a result, most ADP was available to compete with dATP at regulatory sites. In the presence of CDP at a constant concentration of 320 μM, 10-fold higher than the K_m previously reported for CDP with a mammalian RNR (Reichard *et al.*, 2000), ADP did not compete with dATP for binding to regulatory sites on the mR1, resulting in no changes in amount of dATP bound (Figure 5.7, closed circles). Lack of direct effect of ADP on dATP binding to mouse R1 protein was, therefore, confirmed. An interesting result observed from this experiment, however, was the fact that the presence of CDP led to a slight depressive effect on dATP binding.

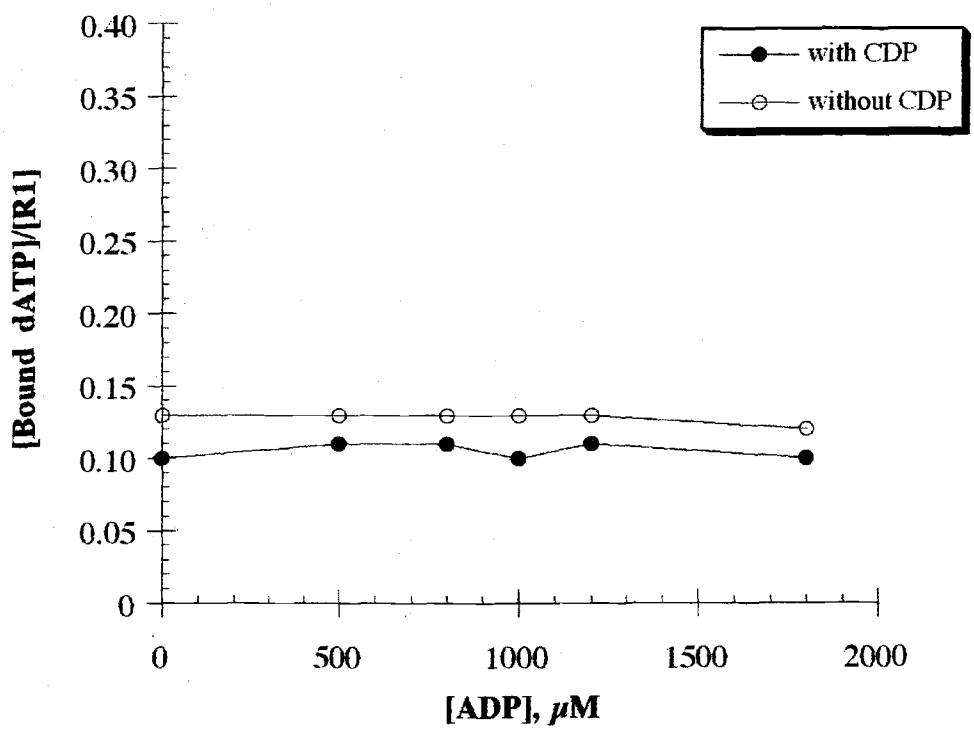


Figure 5.7 Effect of ADP upon dATP binding to mouse R1 protein. In this experiment, both mouse R1 protein and [^{3}H]dATP were present at a final concentration of 1.5 μM. Concentrations of ADP were varied as indicated. Where indicated, CDP was included at 320 μM.

Influence of Ribonucleoside Diphosphates on Binding of Allosteric Modifiers to the Mouse R1 Protein

Although the inhibitory effect of CDP upon binding of dATP was not so pronounced, it was consistent at all concentrations of ADP. Hence, it was speculated that CDP or plausibly all rNDP substrates were capable of directly inhibiting or promoting binding of dNTP allosteric effectors to the specificity site. The depressive effect of CDP was tested by examining the binding of dGTP, an allosteric inhibitor of CDP reduction, in the presence of increasing concentrations of CDP. It was evident that CDP at a concentration as high as 500 μM resulted in a maximal inhibition of dGTP binding to the mR1 protein (Figure 5.8 A, open circles). In fact, dGTP binding was inhibited by nearly 10-fold. GDP, on the other hand, exhibited a stimulatory effect upon binding of dTTP, its prime allosteric activator (Figure 5.8 B, open circles). At a GDP concentration of 500 μM, dTTP binding was increased by 3-fold. These findings suggested direct effects of rNDP substrates on binding of their prime allosteric modifiers to the specificity site on the large subunit of mouse RNR. In addition to the direct effects of CDP and GDP, when a constant concentration of ADP was included along with increasing amounts of CDP or GDP, a distinct effect of ADP upon binding of dGTP and dTTP was discovered. As illustrated in Figure 5.8 A (closed circles), ADP at a concentration of 500 μM, 10-fold higher than the reported K_m for ADP (Reichard *et al.*, 2000) competed efficiently with CDP for binding at the catalytic site. Consequently, the inhibitory effect of CDP upon dGTP binding was modulated, leading to a higher

amount of dGTP bound to the R1 protein. A similar result was obtained when the binding of dTTP was determined as concentrations of GDP were increased in the presence of 500 μM ADP. GDP was essentially displaced by ADP; as a result, its stimulatory effect on dTTP binding was depressed (Figure 5.8 B, closed circles). These data support the idea that binding of ADP at the catalytic site may not directly influence the enzyme specificity. ADP may, in fact, indirectly affect affinities for dNTP modifiers at the specificity site, by competing with other substrates for binding to the catalytic site and in turn depressing the effects of those other substrates upon dNTP binding.

Indirect Effects of ADP upon Binding of Allosteric Modifiers to the Specificity Site on the Mouse R1 Protein

The idea of ADP affecting the enzyme specificity indirectly previously mentioned was tested by determination of dGTP and dTTP binding under various substrate conditions. In Figure 5.9, concentrations of ADP were varied from 0 to 1800 μM, whereas other substrates were present at constant indicated concentrations. As shown in Figure 5.9 A, ADP alone had little or no direct effect on dGTP binding, supporting the result in Figure 5.7. CDP significantly inhibited dGTP binding, compared to results seen when ADP was solely present (circles compared to squares). However, as ADP concentration was elevated, the effect of

CDP was depressed, resulting in a slight increase of dGTP bound (circles). In the presence of the other three substrates—CDP, UDP, and GDP—at their estimated *in vivo* concentrations, dGTP binding was further enhanced (triangles), possibly due to a combination of effects of all rNDP substrates. Similar findings are depicted in Figure 5.9 B. Increasing concentrations of ADP, in the absence of other substrates, did not affect binding of dTTP to the specificity site (squares). GDP remarkably stimulated dTTP binding, but its effect was gradually depressed by increasing concentrations of ADP (circles). A combination of all four rNDPs resulted in the most pronounced inhibition of dTTP (triangles).

New findings reported in this Chapter together, with results presented in Chapter 4, therefore, confirm the two-way communication between the catalytic site and the specificity site on the large subunit of mouse ribonucleotide reductase. It has been known that binding of dNTP modifiers at the allosteric site affects substrate binding at the catalytic site. Yet as shown here, the converse relationship also holds. Certain substrates, such as CDP and GDP, have direct effects upon binding of their prime regulators, dGTP and dTTP, respectively. ADP, in contrast, does not seem to influence dNTP binding directly. It competes with other substrates and displaces them at the catalytic site. As a result, the effects of other rNDPs were modulated accordingly.

Figure 5.8 Effects of rNDP substrates upon binding of allosteric modifiers to mouse R1 protein. A, effect of CDP upon dGTP binding. All binding reaction mixtures contained 6 μM mR1 protein, 6 μM [^{3}H]dGTP, and ADP, where indicated, at 500 μM with varied concentrations of CDP. **B, effect of GDP upon dTTP binding.** All binding reaction mixtures contained 2 μM mR1 protein, 2 μM [^{3}H]dTTP, and ADP, where indicated, at 500 μM with varied concentrations of GDP.

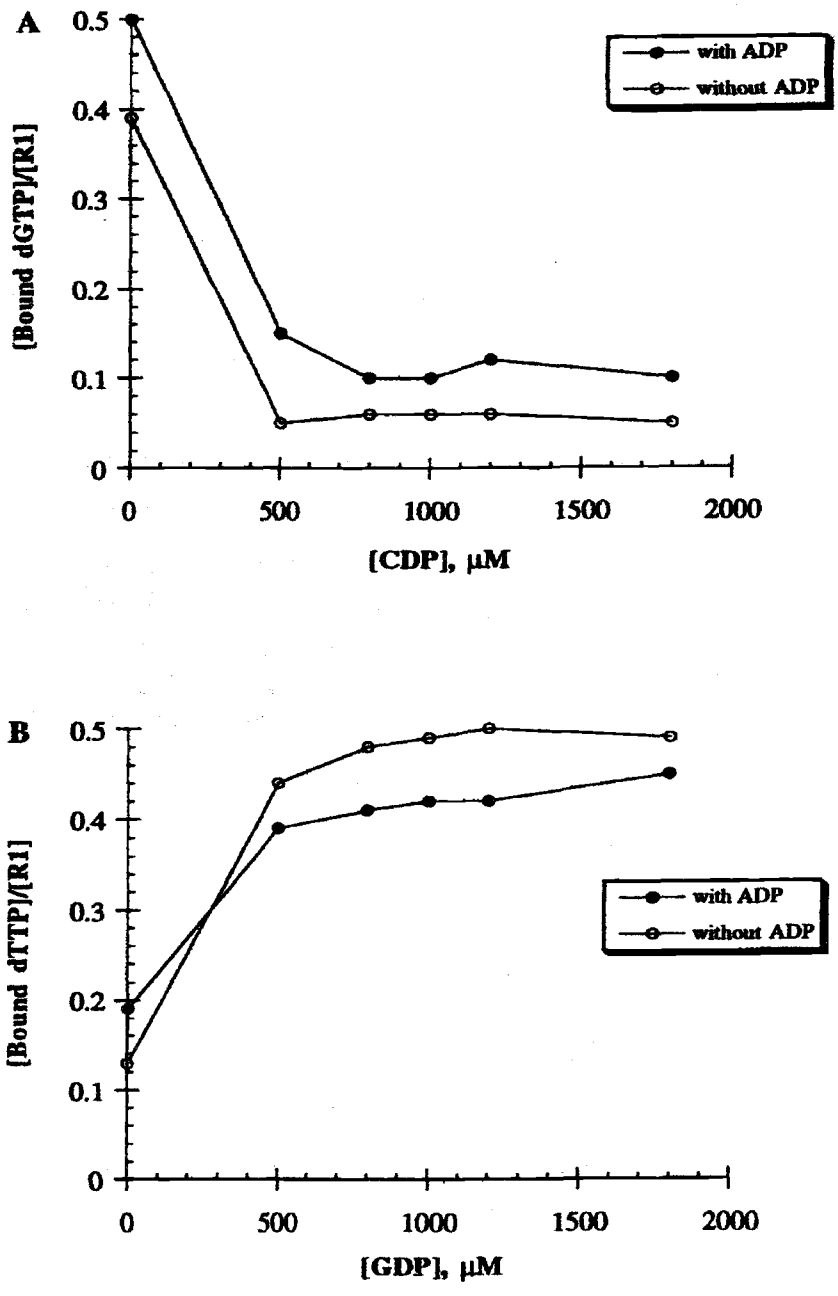
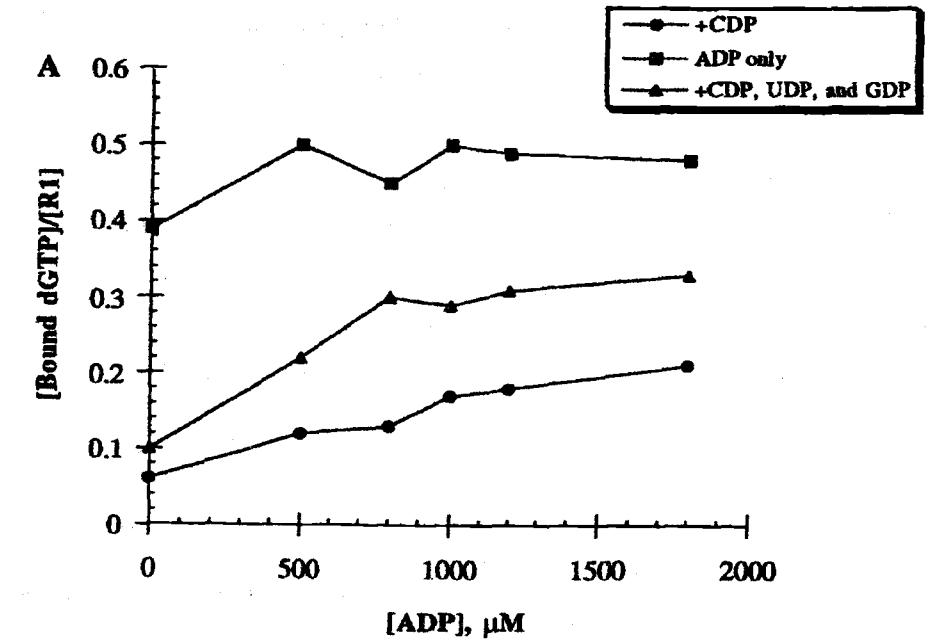

A
0.5
0.4
0.3
0.2
0.1
0
[Bound dGTP]/[R1]
with ADP
without ADP
0
500
1000
1500
2000
[CDP], μM
B
0.5
0.4
0.3
0.2
0.1
0
[Bound dTTP]/[R1]
with ADP
without ADP
0
500
1000
1500
2000
[GDP], μM

Figure 5.8

Figure 5.9 Indirect effect of ADP upon binding of allosteric modifiers to mouse R1 protein. A, dGTP binding. All binding reaction mixtures contained 6 μM mR1 protein, 6 μM [^{3}H]dGTP, and ADP at the indicated concentrations. *Squares*, only ADP present; *circles*, CDP present at 320 μM; *triangles*, 70 μM CDP, 160 μM UDP, and 160 μM GDP were included. **B, dTTP binding.** All binding reaction mixtures contained 2 μM mR1 protein, 2 μM [^{3}H]dTTP, and ADP at the indicated concentrations. *Squares*, only ADP present; *circles*, GDP present at 500 μM; *triangles*, 70 μM CDP, 160 μM UDP, and 160 μM GDP (estimated *in vivo* concentrations) were included.


A
+CDP
ADP only
+CDP, UDP, and GDP
[Bound dGTP]/[R1]
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
[ADP], μM

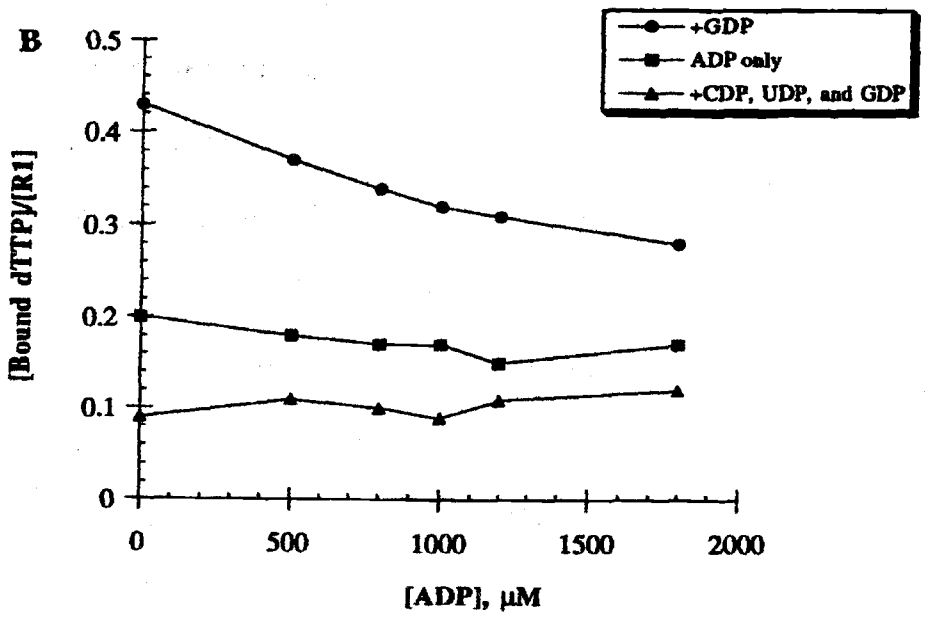

B
+GDP
ADP only
+CDP, UDP, and GDP
[Bound dTTP]/[R1]
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
[ADP], μM

Figure 5.9

Chapter 6

Effect of Limited Oxygen Levels upon Substrate Specificity of Mammalian Ribonucleoside Diphosphate Reductase

Since Class I ribonucleotide reductases can function only under aerobic conditions, allosteric regulation of this type of enzyme has usually been studied *in vitro* at 20% atmospheric oxygen (O_2). However, this percentage of O_2 does not necessarily resemble the O_2 content that mammalian cells sense *in vivo*. In fact, molecular O_2 is present at much lower levels in animal tissues, and cells do occasionally experience varying extents of reduced O_2 supply. Therefore, it is of interest to determine how oxygen limitation affects mammalian RNR specificity as a way to try to understand the role of ribonucleotide reductase in maintaining balanced dNTP levels for DNA replication and repair under specific conditions. Due to the fact that oxygen plays a pivotal role in generating the catalytically essential tyrosyl radical and the oxygen-bridged dinuclear iron center within the small subunit of Class I ribonucleotide reductases (RNRs) (Ochai *et al.*, 1990; Bollinger *et al.*, 1991; Sjöberg, 1997), hypoxia could be another means by which the enzyme is regulated.

Another aspect that makes this investigation worthwhile involves some observations reported by Probst and colleagues. In studies on DNA synthesis of certain tumor cell lines such as Ehrlich ascites (Probst *et al.*, 1988; Brischwein

et al., 1997), lymphoid leukemia CCRF-CEM and Hela cells (Probst *et al.*, 1999), it has been found that regulation of DNA replication depends upon the O_2 in the cellular environment. Under controlled hypoxia (0.02-0.2% O_2), changes in DNA replication initiation are observed along with changes in the dNTP pool sizes. At low O_2, changes in the dCTP pool are pronounced, whereas changes in the dTTP pool are moderate. By contrast, dATP and dGTP levels undergo no changes. Concurrently, a decrease in the intracellular level of the RNR tyrosyl free radical is shown by EPR spectroscopy. Interestingly, a similar observation of dCTP depletion under limited O_2 content has also been made in our laboratory with cultured V79 hamster lung cells (Chimploy *et al.*, 2000, Figure 1). Since RNR is responsible for providing DNA precursors, and operates only in an aerobic environment, Brischwein *et al.* (1997) proposed that RNR may also serve as a pO_2 sensor, transmitting the signal in the form of changes in dCTP levels. In other words, dCTP depletion observed is a result of a distinctive sensitivity of CDP reductase to O_2 tension. With the availability of the four-substrate assay, this chapter will focus on testing this hypothesis.

Determination of the Four Activities of Mouse Ribonucleotide Reductase after Anaerobic Preincubation by Using the Four-substrate Assay

The first approach to investigate the enzyme specificity under hypoxia was to monitor the four activities of the enzyme in the presence of varied O_2 content.

In an attempt to determine the optimal anaerobic incubation time that would lead to enzyme inactivation, a solution of mR1 and mR2 proteins was placed (on ice) in the anaerobic chamber for up to one hour after being bubbled gently with argon gas. At each time point, the two subunits were added to a reaction mixture that contained standard components with an equimolar substrate concentration (0.15 mM), 2.5 mM ATP and 10 μM dTTP. The four-substrate assay was then carried out anaerobically until the reaction was stopped by adding 5 μl of 50% perchloric acid. As shown in Figure 6.1, although the enzyme was anaerobically incubated on ice for up to 60 minutes, at least 50% of all four activities remained. As expected, CDP, GDP and UDP reductase activities were inhibited as the time of incubation increased. However, ADP reductase activity was the most tolerant to anaerobic preincubation. Interestingly, dADP somehow accumulated when the enzyme was anaerobically incubated for 20-40 minutes.

Aerobic Regeneration of Ribonucleotide Reductase Activities after Hydroxyurea Treatment

Since anaerobic preincubation resulted in only 30-50% enzyme inactivation, a different approach was utilized in order to determine the four activities of mouse ribonucleotide reductase under hypoxic conditions. The small subunit (mR2) of the enzyme was first subjected to hydroxyurea (HU) treatment to destroy the tyrosyl radical. The radical was then regenerated under various subatmospheric oxygen

levels after HU removal, leading to regeneration of the four activities which could be monitored by the four-substrate assay. Although HU is known as a drug that targets the free radical on the small subunit of the enzyme, the dosage (used under our assay conditions) that completely inhibits all four reductase activities has not been reported. Thus, it was necessary to determine the optimal concentration of HU that entirely inhibited formation of all four dNDP products. As shown in Table 6.1, HU at a concentration of 20 mM or even 100 mM only partially inhibited the enzyme activity, whereas treatment with 1 M of HU resulted in a complete inactivation.

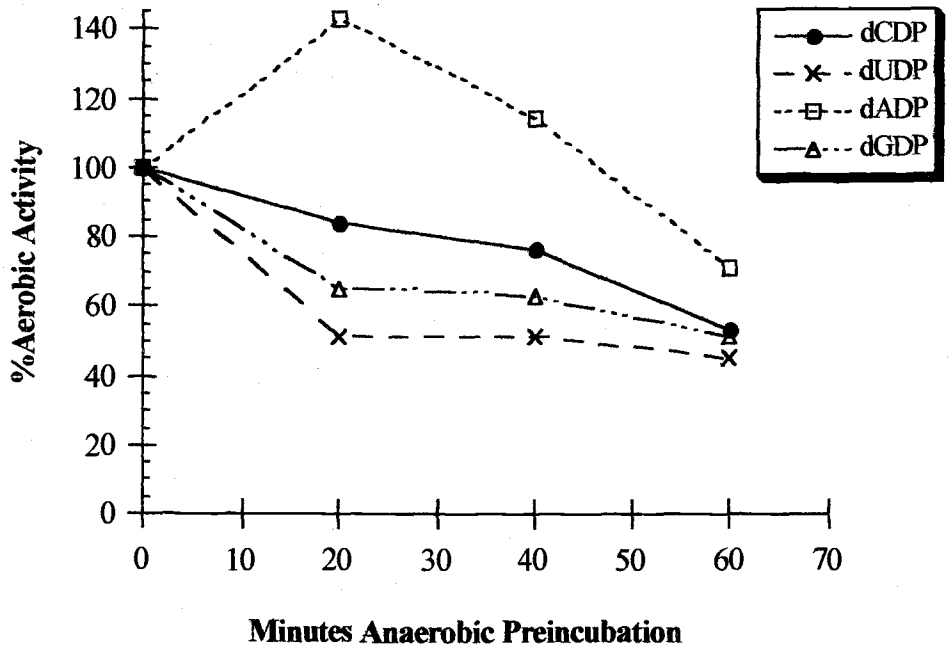


Figure 6.1 Effect of anaerobic preincubation on mouse ribonucleotide reductase's specificity. The four-substrate assay was carried out anaerobically after the enzyme was subjected to anaerobic preincubation. For each indicated time point, the solution of mR1 and mR2 proteins (at final concentrations of 1 μM and 2 μM, respectively) was added to reaction mixtures containing basic components as described previously plus 1.5 mM each of rNDP substrates, 2.5 mM ATP, and 10 μM dTTP. Reactions were allowed to proceed at 37°C for 5 minutes, followed by an addition of 5 μl of 50% PCA, boronate chromatography and HPLC analysis. The four activities after anaerobic preincubation are presented as "% Aerobic Activity", referring to percentages of activities determined under identical but aerobic conditions.

Table 6.1 Effect of hydroxyurea treatment upon the four activities of mouse ribonucleotide reductase. The mR2 protein was incubated anaerobically with various concentrations of HU at 37°C for 10 minutes. Without HU removal, the mR2 solution was added to the reaction mixture containing standard reaction components for the four-substrate assay with estimated *in vivo* concentrations of rNDP substrates and (d)NTP effectors. All reaction mixtures were incubated at 37°C for 5 minutes.

HU concentration (M)	Specific activity (nmol dNDP/min/mg R1)			
	dCDP	dUDP	dADP	dGDP
0.02	8.0	4.0	7.8	2.9
0.10	4.0	2.9	13.6	1.6
1.00	<0.1	<0.1	<0.1	<0.1

Table 6.2 shows the four activities of the enzyme under different regeneration conditions. Aerobic regeneration after the enzyme was anaerobically incubated for 60 minutes on ice without HU treatment resulted in full activity. When the mR2 protein was previously treated with 1M HU, and the radical was regenerated aerobically after HU removal, nearly full activity was regained. In contrast, no detectable activity was restored when the radical was regenerated anaerobically or when HU was not removed from the mR2 protein prior to aerobic regeneration. These results, therefore, indicated that the tyrosyl radical on the small subunit of mouse ribonucleotide reductase could be completely destroyed by

1 M HU and regenerated under an aerobic condition (20% O_2) to nearly full activity. This approach was then chosen as a suitable alternative to measure the enzyme activity under varied oxygen levels.

To examine effects of lowered oxygen levels upon the four activities of ribonucleotide reductase, the four-substrate assay was performed in the presence of O_2 at 0, 0.5, 1.5, 3 and 20 %. The R2 protein used in all reactions was first anaerobically incubated with 1 M HU at 37°C for 10 minutes. After HU was removed by the use of a Centri-Sep spin column, the HU-free R2 protein was added to each reaction mixture at a final concentration of 2 μM. Regeneration and reaction were allowed to proceed by an incubation at 37°C for 5 minutes inside the anaerobic chamber saturated with an anaerobic gas mixture (5% H_2, 5% CO_2, 90% N_2) and an indicated amount of oxygen. During the incubation period, reaction mixtures were gently bubbled with the same gas mixture as that present inside the chamber to ensure maximal exposure to indicated percentages of O_2. For regeneration at 20% O_2, the reaction was carried out aerobically. As illustrated in Figure 6.2, all four activities of mouse ribonucleotide reductase were inhibited as the percentage of O_2 was decreased. Interestingly, the four activities were differentially affected by oxygen limitation with varied degrees of sensitivity. Results from two independent experiments showed that UDP reductase activity was the most tolerant, whereas GDP reductase were the most sensitive. CDP and ADP reductase activities exhibited intermediate sensitivity to hypoxia.

Table 6.2 Aerobic regeneration of mouse ribonucleotide reductase activities after hydroxyurea treatment. Except for the typical assay, all reaction components were assembled inside the anaerobic chamber. After the mouse R2 protein was incubated anaerobically with 1 M hydroxyurea for 10 minutes at 37°C, the solution was placed on ice. Hydroxyurea was immediately removed by a Centri-Sep spin column. The HU-free R2 protein was then added to a final concentration of 2 μM to the reaction mixture containing 1 μM R1, 20 μM ferrous ammonium sulfate, 50 mM dithiothreitol, and rNDP substrates and allosteric effectors at their estimated *in vivo* concentrations. For aerobic regeneration, the reaction was carried out outside the chamber for 5 minutes at 37°C after being gently bubbled with air. In contrast, the "anaerobic regeneration" reaction was bubbled with anaerobic gas mixture and incubated inside the chamber. "Typical assay" refers to the standard four-substrate assay carried out aerobically as described in Chapter 3.

Treatment conditions	Product formation (nmol/min/mg R1) ± SD			
	dCDP	dUDP	dADP	dGDP
Typical assay	18.8 ± 2.7	8.7 ± 0.9	8.6 ± 0.2	5.7 ± 0.2
Regeneration in air, no HU treatment	15.7 ± 3.9	7.7 ± 0.5	7.3 ± 1.6	5.2 ± 1.1
HU treatment, aerobic regeneration	12.9 ± 1.0	7.0 ± 0.2	6.8 ± 1.1	4.9 ± 0.6
HU treatment, anaerobic regeneration	<0.1	<0.1	<0.1	<0.1
No HU removal, aerobic regeneration	<0.1	<0.1	<0.1	<0.1

Note: HU = hydroxyurea
SD = standard deviation

These data did not agree with the hypothesis that depletion of dCTP at low O_2 level was caused by the special sensitivity of CDP reductase activity (Probst *et al.*, 1988). In fact, as oxygen levels became limited, GDP reductase appeared to be the most sensitive. At 1.5% O_2, 75% of GDP reductase activity was inhibited while ADP, CDP and UDP reductases were 46, 35, and 31%, respectively repressed. An additional experiment was carried out to examine whether regeneration of GDP reductase activity was slower than that of other three activities, resulting in being the most sensitive to 1.5% O_2 after a 5-minute incubation period. The assay conditions were identical to Figure 6.2 except that in this experiment, the assays were performed only at 1.5% O_2 with the regeneration time of 5, 10, and 15 minutes. Evidently, even though regeneration was allowed to proceed for up to 15 minutes, the product profile remained similar to that seen after 5 minutes (Figure 6.3). In comparing the product formation of the two time points, a 5-minute incubation period resulted in 18% dADP, 49% dCDP, 8% dGDP, and 25% dUDP, whereas 29, 36, 12, and 23% of dADP, dCDP, dGDP, and dUDP, respectively were formed after 15 minutes. Hence, regeneration of GDP reductase activity was rapid, but perhaps incomplete during 5 minutes of regeneration under lowered O_2 levels.

Another interesting aspect to explore was whether the differential response to oxygen limitation seen in Figure 6.2 was due to variations in allosteric configurations of the enzyme. With ATP present as a primary activator (bound

to the activity site), effects of two dNTP modifiers (bound to the specificity site)—dTTP and dGTP—were examined in the presence of 1.5% O_2 (Figure 6.4). Figure 6.4A indicated that binding of dTTP at the specificity site of the mouse RNR's large subunit led to a remarkable increase of GDP reduction. When only dGTP and ATP were present as allosteric effectors, reduction of ADP (and GDP) were augmented (Figure 6.4B). Results from these two experiments suggested that allosteric regulation of the enzyme by dNTPs under hypoxic and aerobic conditions was essentially similar. Therefore, different allosteric forms of the enzyme did not contribute to differential effects of oxygen depletion upon the four reductase activities.

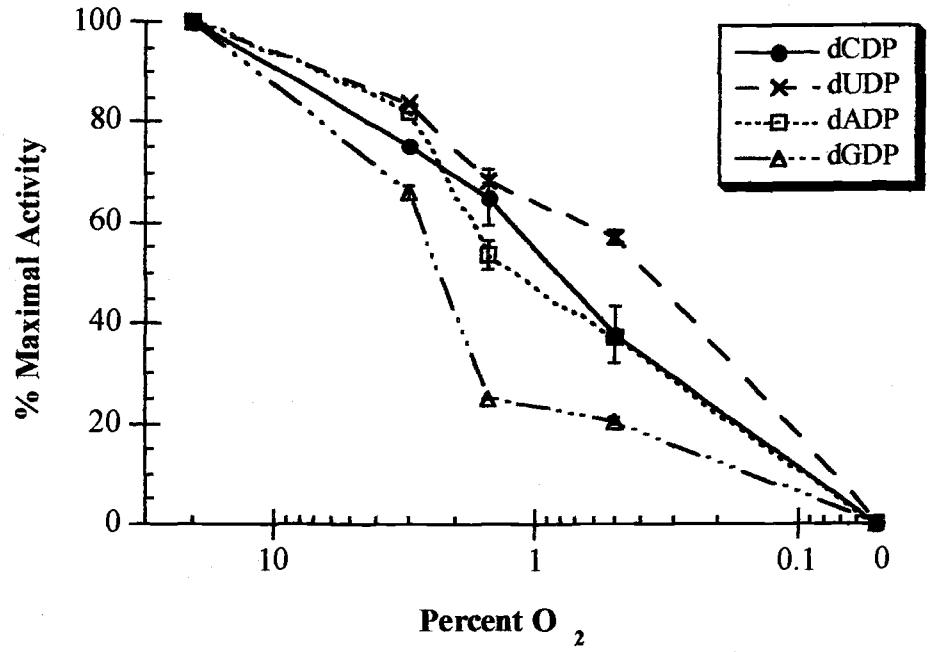


Figure 6.2 Influences of limited oxygen levels on mouse rNDP reductase activities. The RNR small subunit (R2) was treated with 1 M HU at 37°C for 10 minutes. After HU removal, the HU-free R2 protein was added at a concentration of 2 μM to the reaction mixtures containing essential reaction components and 1 μM R1. All rNDP substrates and allosteric effectors were present at their estimated physiological concentrations. Regeneration and reaction were carried out at indicated oxygen levels for 5 minutes at 37°C. The data are presented as percentages of the respective activities regenerated aerobically (20% O_2) versus Percent O_2. Activities shown are averaged values obtain in two independent experiments.

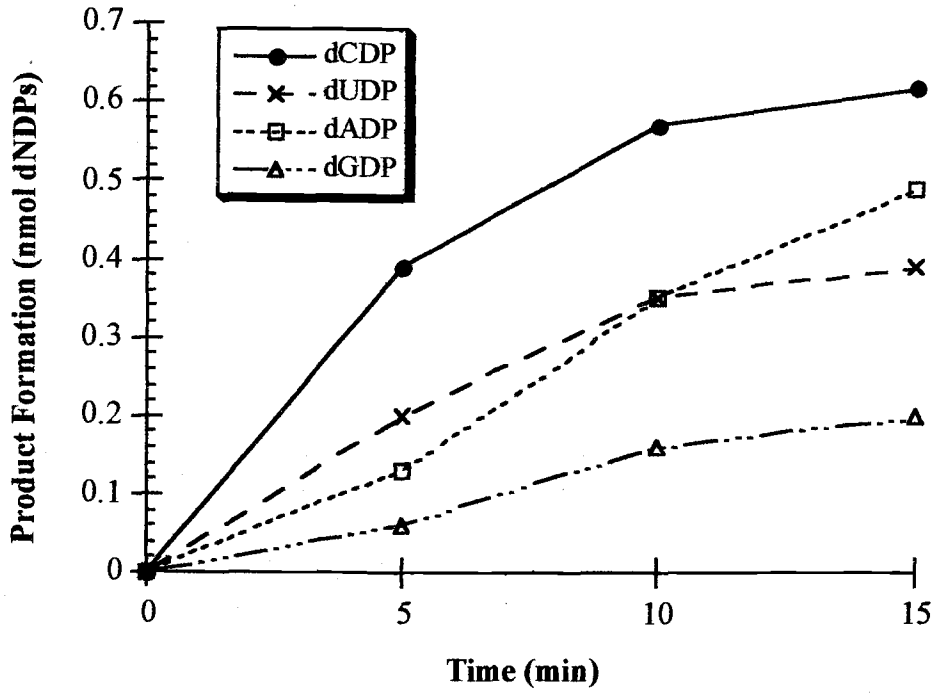


Figure 6.3 Effects of regeneration periods at 1.5% O_2 upon rNDP reductase activities. The four-substrate assay was performed in the presence of 1.5% O_2 with estimated *in vivo* concentrations of substrates and allosteric modifiers. Regeneration and reaction were allowed to proceed at 37°C, and samples were taken at the indicated times. At each time point, the sample was processed as previously described (Chapter 3) by using boronate affinity chromatography followed by HPLC analysis.

Figure 6.4 Effects of dNTPs upon the four activities of mouse ribonucleotide reductase during regeneration at 1.5% O_2. In this experiment, the regeneration and reaction were carried out in 1.5% O_2 in the presence of only 2.5 mM ATP and 50 μM dTTP (A) or only 2.5 mM ATP and 15 μM dGTP (B) as allosteric effectors. All four dNDP substrates were present at their approximate *in vivo* concentrations.

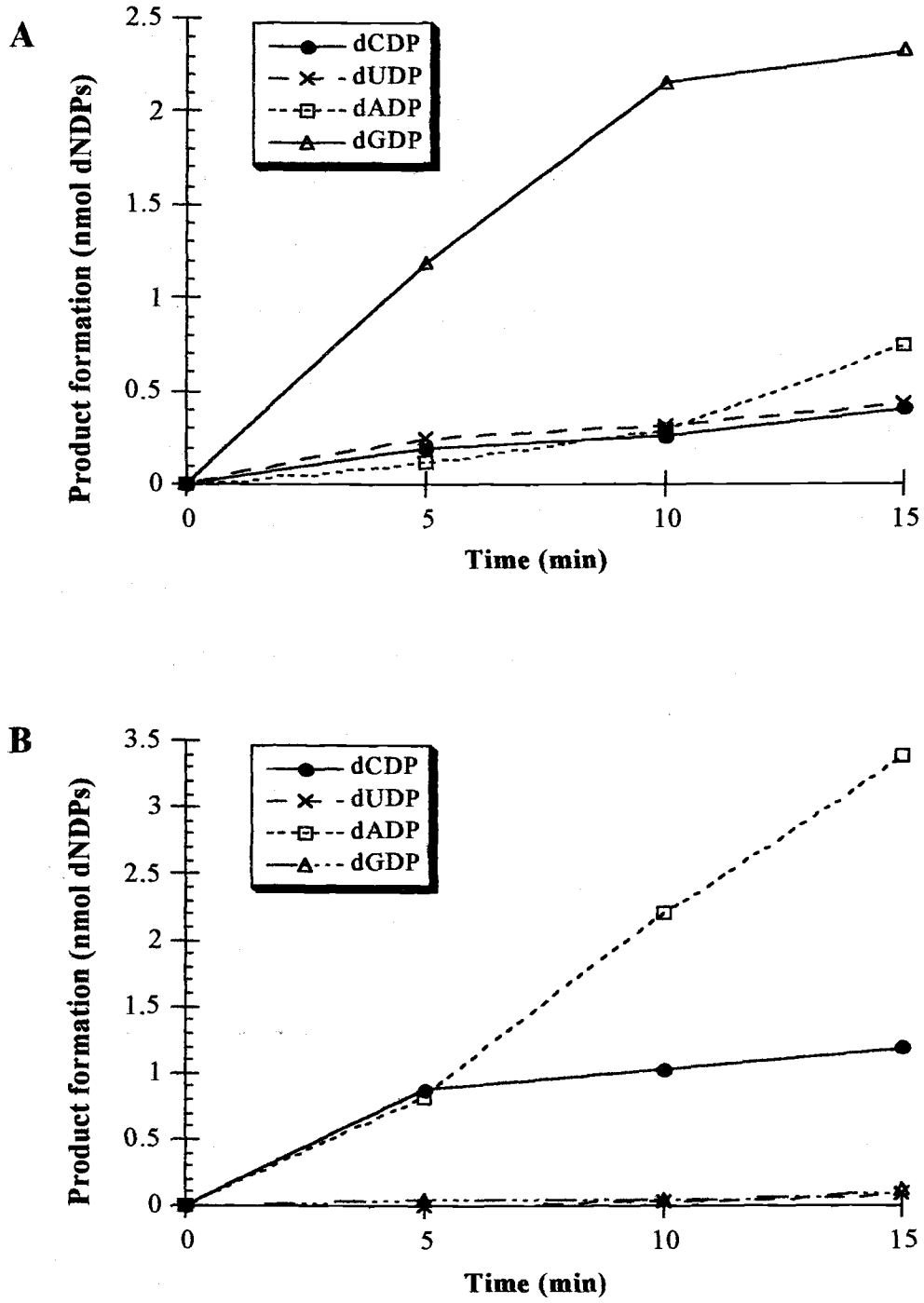

A
Product formation (nmol dNDPs)
2.5
2
1.5
1
0.5
0
dCDP
dUDP
dADP
dGDP
0
5
10
15
Time (min)
B
Product formation (nmol dNDPs)
3.5
3
2.5
2
1.5
1
0.5
0
dCDP
dUDP
dADP
dGDP
0
5
10
15
Time (min)

Figure 6.4

Chapter 7

Discussion and Conclusion

Overexpression and Purification of Recombinant Mouse Ribonucleotide Reductase Subunits

The genes encoding the large (R1) and small (R2) subunits of mouse (from the Thelander laboratory) and vaccinia virus (available in the our laboratory) ribonucleotide reductases (RNRs) have previously been cloned into pET vectors, which are under the control of T7 promoter. Thus overexpression of corresponding proteins can be induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) under conditions described in Chapter 3. Procedures for purifying R1 and R2 proteins, however, are essentially different. Purification of the R1 protein is carried out by using a dATP-Sepharose column, a one-step affinity chromatography. Since dATP binds more tightly to the R1 than ATP does, most of the purified protein is eluted in a dATP-containing fraction (85.3% of total protein). Before utilizing the protein in the four-substrate RNR assay, separation of ATP or dATP from the R1 is always considered to ensure accurate total amounts of these nucleotides in reaction mixtures. Due to the low binding affinity of ATP, ATP can be easily removed by replacement with an excessive amount of buffer over a Centricon-30 centrifugal filter device. Removal of dATP, on the other hand, is relatively difficult because

of its high affinity for R1. Hence, treatment with 1 M NaCl, followed by separation on a Centri-Sep spin column is routinely and successfully used.

Purification of the R2 protein is composed of four major steps—streptomycin sulfate precipitation, ammonium sulfate precipitation, gel filtration chromatography and strong anion exchange chromatography. Including the step of tyrosyl free radical and dinuclear iron center regeneration, the entire process to obtaining only partially purified R2 usually takes a few days. Although further fractionation may help remove some contaminants, the risk of losing enzyme activity via protein degradation and/or denaturation caused by extended exposure of the enzyme to repetitive procedures at non-optimal temperature is undesirable. To make sure that the protein is of high enough quality for our purposes of investigation, it has been confirmed that the purified fraction does not contain critical enzymes such as *E. coli* host's RNR or nucleoside diphosphate kinase (NDPK), which could interfere with determination of rates of product formation generated by mouse RNR, our enzyme of interest.

The Reducing Agent Utilized in the Four-Substrate Assay

The four-substrate assay is usually performed in the presence of dithiothreitol (DTT) as an electron supplier. In fact, DTT has been utilized routinely *in vitro* for enzymatic studies of T4 bacteriophage, *E. coli*, vaccinia virus, mouse, and potential mammalian mitochondrial RNRs in our laboratory, even

though there is evidence of higher efficiency achieved when DTT is replaced by the protein-based redox systems. It has been reported that the rate of CDP reduction by T4 bacteriophage RNR with DTT present is only 10% of the rate obtained when glutaredoxin is used, reflecting lower efficacy of DTT to serve as a reductant (Berglund, 1972). In Chapter 4, depicted in Figure 4.6 and Table 4.3, we have measured the four activities of mouse RNR with DTT or thioredoxin present as reducing agents. In comparison, it is evident that there is no significant difference in overall product profiles between using DTT and thioredoxin as reductants during 5 minutes of incubation, our typical reaction time. Therefore, DTT is proven to be a convenient yet appropriate reducing system under our assay conditions. Nonetheless, the fact that dGDP formation in the presence of thioredoxin, a reducing agent found *in vivo*, is approximately two-fold lower than that in the presence of DTT (shown in Table 4.3) is noteworthy. Via a different mechanism from DTT, thioredoxin may contribute, at least partly, to dGTP under-representation inside the cell. Or DTT may lead to overestimation of the amount of dGDP (and thus of dGTP) actually formed *in vivo*. However, in order to understand mechanisms by which the two reductants affect rates of product formation by mouse ribonucleotide reductase, further investigations are necessary.

Effects of Individual Allosteric Modifiers on the Four Activities of Mouse Ribonucleotide Reductase

In Chapter 4, it has been confirmed that, like other Class I enzymes, binding of each nucleoside triphosphate modifier at the specificity site has differential effects on the four activities of mouse ribonucleotide reductase. ATP is required for the overall activity of mouse ribonucleotide reductase. As shown in Figure 4.8 (Chapter 4), the enzyme becomes basically inactive in the absence of ATP, indicating a critical role of ATP as a primary modulator. Likewise, a non-hydrolyzable ATP analog, 5'-adenylylimidodiphosphate (AMP-PNP) can also serve as a general activator for mouse RNR. However, it does not stimulate the enzyme in the same manner as ATP does, as it results in different product profiles. Hence, this ATP analog has unique regulatory effects upon the four activities of RNR. This result is in an agreement with the results obtained from *E. coli* and vaccinia virus RNRs (Hendricks Ph.D. thesis, 1998 and SPH notebook #7, page 20). It is interesting, nonetheless, that reduction of ADP by *E. coli* and vaccinia virus RNRs is completely inhibited when AMP-PNP is present as a single regulator; whereas the rates of formation of the other products are just about the same as the rates measured in the presence of ATP. A similar inhibitory effect of AMP-PNP upon ADP reductase activity has been observed with mouse RNR. That is, the rate of dADP formation in the presence of AMP-PNP is four times lower than that in the presence of ATP (Table 4.4, Chapter 4). However, additional effects of AMP-PNP limited to the mouse RNR have also been discovered. First,

reduction of UDP is at least two-fold decreased when AMP-PNP is used as an allosteric modifier. And second, GDP reduction is not strictly dependent on dTTP, unlike the case when only ATP is present. The mechanism by which AMP-PNP regulates the four activities of RNRs is not known. Yet it is possible that binding of AMP-PNP at the activity site and/or the specificity site leads to conformational changes that favor distinct substrate preferences. Moreover, since it contains only one phosphate, AMP-PNP may be able to mimic ADP and bind to the catalytic site, replacing ADP and thus in turn, inhibiting ADP reduction. It should be noted, otherwise, that when other allosteric effectors are included in the reaction mixture, influences of AMP-PNP are in accord with those of ATP (Table 4.5, Chapter 4). Besides, addition of allosteric effectors in the presence of either AMP-PNP or ATP results in rates of product formation that are closer to the nucleotide composition of the mouse genome than the case when only either AMP-PNP or ATP are solely present. This suggests that the proportional combination of dNTP modifiers keeps the enzyme in a configuration that favors proportionate reduction of all four substrates, overcoming the inhibitory effects of AMP-PMP. In other words, the conformation of the R1 protein with dNTP bound at the specificity site does not allow binding of AMP-PNP at the catalytic site (if AMP-PNP is, indeed, capable of binding at the particular site). In addition, the ability of AMP-PNP to cause the conformational change is limited by dNTP binding; as a result, AMP-PNP can only bind and activate the enzyme in the same manner as ATP does.

Allosteric behavior of mouse RNR in response to other dNTPs, namely, dCTP, dGTP and dTTP is comparable to that of other mammalian RNRs described earlier (Eriksson *et al.*, 1979 and Reichard *et al.*, 2000). It is evident that dCTP does not play any role as an allosteric regulator since it has no effect upon product formation by the mouse RNR. As expected, dGTP activates primarily ADP reduction and inhibits pyrimidine nucleotide reduction. dTTP is required for reduction of GDP, but its effect on pyrimidine nucleotide reduction is inhibitory. In addition to the effects mentioned above, dGTP also activates GDP reduction; and dTTP stimulates ADP reduction, although to a lesser extent.

Inhibitory effects of dATP upon mouse RNR activity depend on the total amount of dATP in the reaction mixture. As illustrated in Chapter 4, Figure 4.10 panel D and Table 4.6, dATP present at 40 μM binds significantly to the activity site, resulting in a small stimulatory effect on ADP reduction and a strong inhibitory effect on CDP and UDP reduction. At higher concentrations (100 μM), all four activities are inhibited. Among eukaryotic RNRs, the virus-induced RNR is reported to be more resistant to effects of dATP than its mammalian counterparts. In fact, only 50% of the activity in vaccinia virus-infected cell extracts is inhibited when dATP at 100 μM is present (Slabaugh *et al.*, 1984). On the other hand, nearly 60% of the control activity of calf thymus RNR is repressed in the presence of dATP at a concentration as low as 10 μM (Eriksson *et al.*, 1979). With the mouse enzyme, approximately 80% and 95% of the activity is inhibited in the presence of dATP at 20 μM and 100 μM, respectively (Figure 4.13, Chapter 4). Apparently,

a lower amount of dATP is required in mammalian cells in order to obtain the same extent of inhibition as in virus-infected cells. This may suggest that mammalian RNRs are more tightly regulated *in vivo*.

As shown in Chapter 4, the allosteric control of mouse RNR by individual dNTPs generally is similar to that of other Class I enzymes. However, some allosteric behaviors discussed below are limited to only the mouse enzyme analyzed by the four-substrate assay.

1) The enzyme activity is ATP-dependent. In the absence of any nucleoside triphosphate, the enzyme is completely inactive.
2) GDP reductase activity is absolutely dependent upon dTTP. As observed repeatedly in the four-substrate assay, formation of dGDP is non-detectable when dTTP is omitted.
3) Compared to vaccinia virus RNR, the mouse enzyme seems to be more sensitive to allosteric modifiers. For instance, the inhibitory effect of dGTP on pyrimidine nucleotide reduction is greater for the mouse enzyme than the viral counterpart. Additionally, GDP reductase activity of the viral RNR does not require dTTP. In fact, added dTTP results in only a two-fold increase in dGDP formation. Besides these discrepancies, the mouse enzyme also shows higher activity with UDP as a substrate.

4) The activation of ADP reduction by dTTP and the activation of GDP reduction by dGTP are not seen in allosteric regulation of calf thymus RNR or mouse RNR analyzed by a single-substrate assay.

Variations in the Intracellular Concentrations of Allosteric Modifiers

Although determination of the effect of each allosteric modifier gives us some valuable ideas of how a mammalian form of such a pivotal enzyme as mouse RNR is allosterically regulated, the enzyme is not subjected to one single effector at a time. All four nucleoside triphosphates are present inside the cell all at the same time and ensure proportional production of all four dNDPs. Hence, it is beneficial to investigate the response of the mouse RNR to all four allosteric modifiers in one reaction mixture. As expected, variations in concentrations of dNTP modifiers significantly influence the product profile of the enzyme. As the amount of the proportionally varied effector mixture increases, differential effects upon the four activities of the enzyme become manifest (Figure 4.15, Chapter 4). It possibly is coincidental yet interesting that the product profile obtained from this experiment seems to be the converse of that from the experiment in which the rNDP substrates were proportionally varied. In Figure 4.15, the relative rate of CDP reduction continuously decreases, whereas the rates of ADP and GDP reduction slightly increase as concentrations of effectors increase. UDP reductase

activity increases only until the concentrations of the modifiers reach their estimated *in vivo* levels then becomes constant.

The fact that UDP reductase seems to be the most tolerant to changes in levels of dNTP effectors (Figure 4.15, Chapter 4), rNDP substrates (Figure 5.1, Chapter 5) or even O_2 tensions (Figure 6.2, Chapter 6) has caught my attention. Studies on vaccinia virus and T4 bacteriophage RNRs have shown that the production of dUDP is consistently low, indicating that dUDP is not a major precursor for dTTP synthesis (Hendricks Ph.D. thesis, 1998). It is reported that *in vivo* the major pathway for the synthesis of dTTP is likely to involve dCMP deamination (dCMP → dUMP → dTMP → → dTTP) rather than the pathway via RNR (UDP → dUDP → dUTP → dUMP → dTMP → → dTTP) (Reichard, 1988). This is supported by the evidence showing that a severe blockage of thymidylate synthase (catalyzes dUMP → dTMP) leads to dUTP accumulation in the cell. As a result, excessive dUTP can be incorporated into DNA, causing an event called "thymineless death" (Ingraham *et al.*, 1986). However, analysis of the mouse RNR shows relatively high activity of the enzyme toward UDP, suggesting that the bypass of dUTP formation to avoid dUTP accumulation may not be the case for mammalian enzymes. Or there may be additional factors that regulate UDP reductase activity *in vivo*. As mentioned earlier, resistance of UDP reductase activity to allosteric environmental changes may have some biological significance. Since dTTP is strictly required for GDP reduction by mouse RNR, perhaps dUDP is also a crucial source for dTTP production in order to maintain an adequate dTTP

pool for GDP reduction as well as to serve as a building block for DNA replication and repair. If the dCMP deamination-dependent pathway somehow becomes inhibited, the other pathway can still provide an ample supply for dTTP synthesis. This means that the dTTP synthetic pathway, involving RNR, is equally essential as the one via dCMP deamination for mouse RNR. Even though the ideas are not too farfetched, it remains to be determined whether dUDP is a critical player in thymine metabolism, or whether there are any other factors involved in regulation of UDP reductase activity.

In an attempt to understand allosteric control of mouse RNR under quasi-physiological conditions in more detail, several additional experiments have been carried out. The decrease of CDP reduction when allosteric regulators are proportionally present is apparent. To identify which dNTP(s) exerts its inhibitory effect upon CDP reductase activity, the mouse enzyme has been assayed under various allosteric environments with variations of each effector's concentration. As shown in Figure 4.17, Chapter 4, dATP does not seem to be responsible for the decrease of CDP reduction. In fact, at low concentration, dATP activates reduction of pyrimidine nucleotides and inhibits reduction of purine nucleotides, consistent with results seen in previous studies on RNR allosteric control. However, as the dATP concentration rises, presumably the binding of dATP also happens at the activity site, relative rates of product formation remain essentially unchanged. Figures 4.17, 4.19 and 4.20 reveal that dTTP and dGTP, at least partly, contribute to inhibition of CDP reductase activity. Interestingly, the effects of dTTP upon the

enzyme specificity, in the presence of other allosteric modifiers and four substrates, are distinct (Figure 4.18). Reduction of CDP and GDP (to a lesser extent) is activated as the concentration of dTTP increases. In contrast, reduction of UDP and ADP (slightly) is inhibited. This result, together with the ones seen when dATP and dGTP are varied in the presence of "*in vivo*" concentrations of substrates and modifiers, suggests that all substrates and allosteric effectors may be responsible for the product profile obtained in Figure 4.15. This speculation has been supported by other evidence demonstrating that dCTP in the presence of the same quasi-physiological condition has differential effects on the four activities (Figure 4.22). However, if dCTP is present as the sole modifier, the product profile undergoes no changes as dCTP concentrations are varied (Figure 4.21). It has been proven in Chapter 5 that rNDP substrates do influence binding of dNTP to the specificity site on R1, which, in turn, alter the relative rates of product formation.

Influence of Substrate Binding upon Interactions with Allosteric Effectors

In vivo, RNR is exposed to a proportionate combination of nucleotides—both rNDP substrates and allosteric effectors. When the enzyme is assayed under different allosteric environments, it is evident that rNDP substrates are equally important as dNTP modifiers in determining the relative rates of product formation. As shown in Table 5.1 of Chapter 5, the rates of product formation are the closest to the rates of corresponding nucleotide utilization when substrates and effectors

are present at their estimated physiological concentrations. Reduction of ADP and UDP corresponds closely to the proportion of dAMP and dTMP, respectively in the mouse genome. Again, the high activity of UDP reductase agrees with the speculation that dUDP may be another significant source for dTTP synthesis, in this case. Nonetheless, the higher rate of dCDP production than the rate of utilization confirms that dCMP deamination is still a major route for dTTP production.

In Chapter 5, it has been shown that rNDP substrates also play an important role in controlling the enzyme specificity. As the amount of proportional substrate mixture increases, differential effects upon the four activities become evident (Figure 5.1). Reduction of ADP and GDP is inhibited, whereas CDP reduction is activated by high concentrations of the substrate mixture. Variations in concentrations of each substrate individually reveal that ADP at high concentrations exerts the same effects seen in Figure 5.1, although it is less pronounced (Figure 5.3). Variations in CDP, GDP and UDP concentrations indicate a simple competition among substrates in binding to the catalytic site (Figure 5.2). The regulatory role of ADP, inhibiting its own reduction has also been observed with vaccinia virus RNR; however, inhibition of GDP reduction and activation of CDP reduction are limited to the mouse enzyme.

Ultrafiltration nucleotide binding assay has been utilized to further determine the effects of rNDP substrates upon binding of the dNTP allosteric modifiers. Binding constants (K_d) of dTTP and dGTP are 1.8 ± 0.30 μM and

4.4 ± 0.38 μM, respectively. Since dATP binds to two different sites, its K_d values for the high affinity site and the low affinity site are 12.6 ± 2.3 μM and 54.3 ± 4.0 μM, respectively (Chimploy, 2001). The values reported by Reichard *et al.* (2000) are 0.07 and 1.5 μM for dATP and 0.2 μM for dGTP. Apparently, our values are notably higher without a known basis for the differences. However, it should be noted that our numbers are closer to the actual intracellular concentrations of corresponding allosteric modifiers determined by our own laboratory with different mammalian cell lines (Leeds et al., 1985) and estimated by Traut (1994) (24 μM for dATP and 5.2 μM for dGTP). Studies on dNTP binding in the presence of various substrate environments indicate some significant properties of rNDP substrates;

1) There is a competition among rNDP substrates for the catalytic site, as clearly shown in Figures 5.2 and 5.7-5.9, Chapter 5.
2) Binding of one substrate at higher concentrations at the catalytic site affects the binding affinity of a certain dNTP at the specificity site, resulting in a change in the enzyme specificity.

   2.1) Substrates like CDP and GDP have direct impacts on dNTP binding. CDP inhibits binding of dGTP, its primary inhibitor, whereas GDP enhances binding of dTTP, its primary activator.

2.2) ADP seems to have a unique feature, instead of a direct effect. That is, it is capable of displacing other substrates at the catalytic site and, in turn, modifying the affinity of the enzyme for dNTPs at the specificity site.

3) Effects of rNDP substrates are concentration-dependent. For instance, the higher the concentration of GDP, the higher the amount of dTTP bound to the specificity site.

Proposed Models for Effects of rNDP Substrates upon Interactions with Allosteric Modifers

Two models are proposed here to explain potential mechanisms by which RNR specificity is regulated. These models, based on the results presented above, give some ideas of how rNDP substrates control the four activities of mouse RNR. Whether or not they represent the actual events taking place in the cell, the models need to be tested.

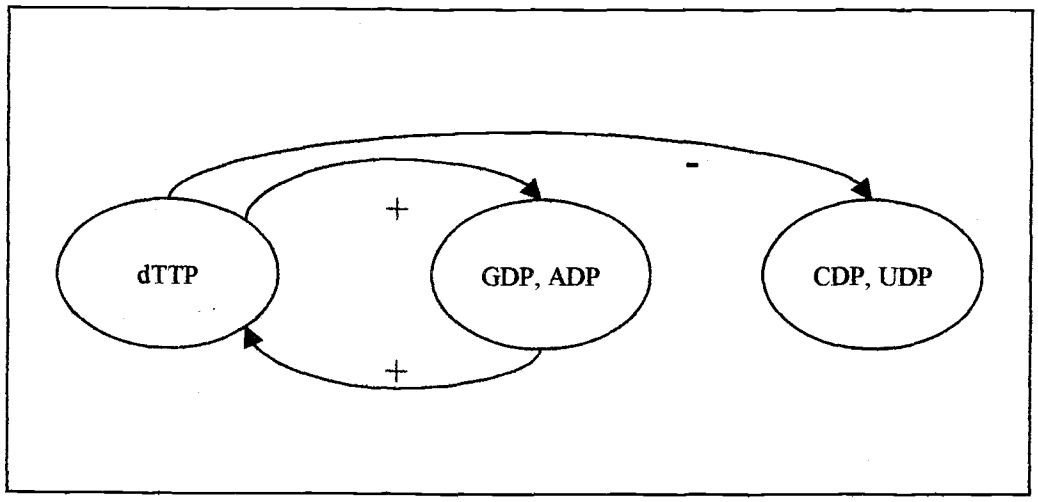
Model A shows a positive effect of GDP and a negative effect of ADP on dTTP binding. Under normal conditions (balanced rNDP levels), GDP promotes dTTP binding at the specificity site. In turn, dTTP then activates reduction of ADP and GDP yet inhibits reduction of pyrimidine nucleotides. As substrate environments become imbalanced (ADP accumulation while pyrimidine nucleotide levels become low, in this case), ADP at a high concentration can effectively

compete with GDP in binding to the catalytic site. Loss of GDP binding at the catalytic site results in a conformational change of the specificity site, which in turn affects dTTP binding. A decrease in dTTP binding, thus, decreases reduction of purine nucleotides, but elevates reduction of CDP and UDP. This model fits our previous observation that ADP (at a high concentration) inhibits its own reduction and that of GDP, but activates CDP reduction.

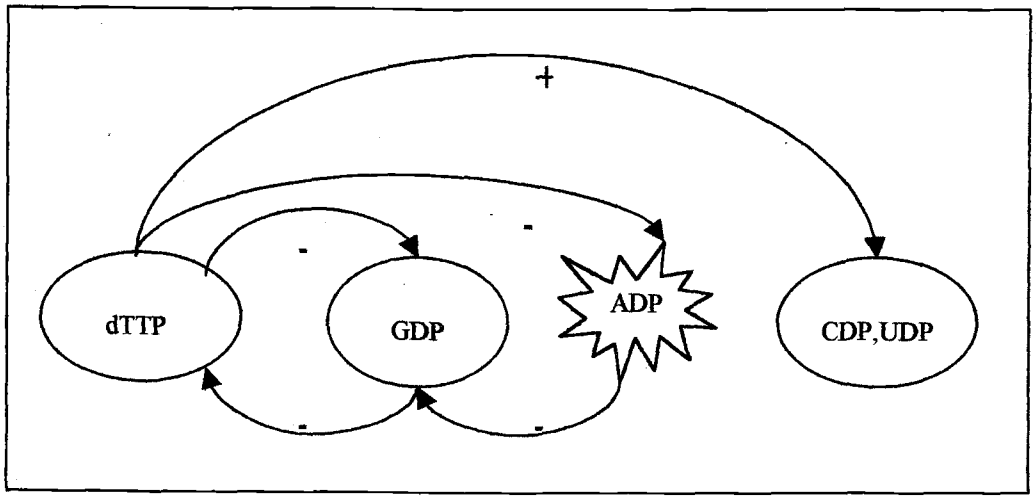
Model B depicts a negative effect of CDP and a positive effect of ADP upon dGTP binding. CDP inhibits the binding of dGTP, which normally inhibits reduction of CDP and UDP, but activates reduction of ADP and GDP. As rNDPs contents are imbalanced, for example, when the intracellular ADP concentration is too high, ADP then competes with CDP, displacing it from the catalytic site. Consequently, the inhibitory effect of CDP is removed. The conformation of the enzyme now favors dGTP binding, leading to activation of ADP (and GDP) reduction and inhibition of CDP (and UDP) reduction. Since reduction of ADP has been activated, the level of ADP will then decrease, resulting in balanced levels of rNDP substrates again.

Model A dTTP Binding

1) Balanced rNDPs

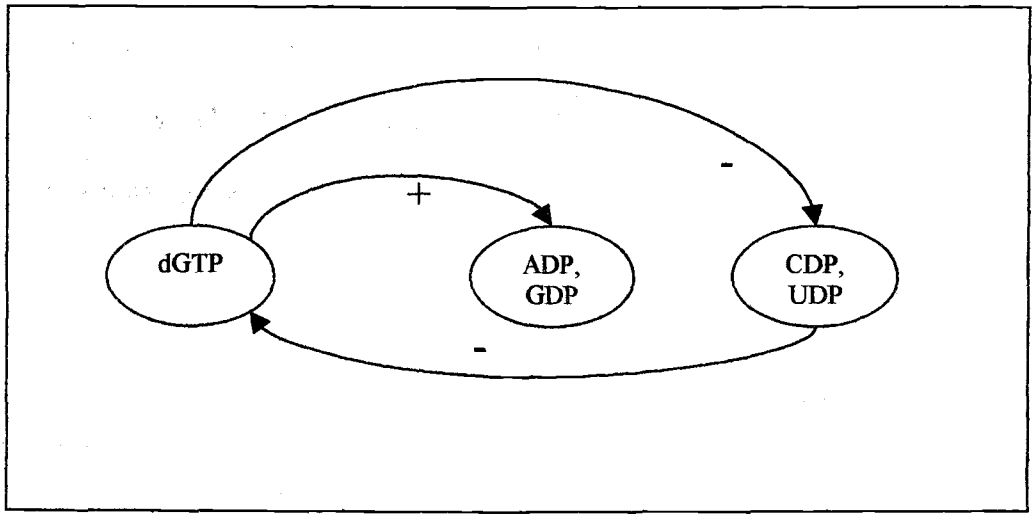


2) Imbalanced rNDPs (High [ADP])

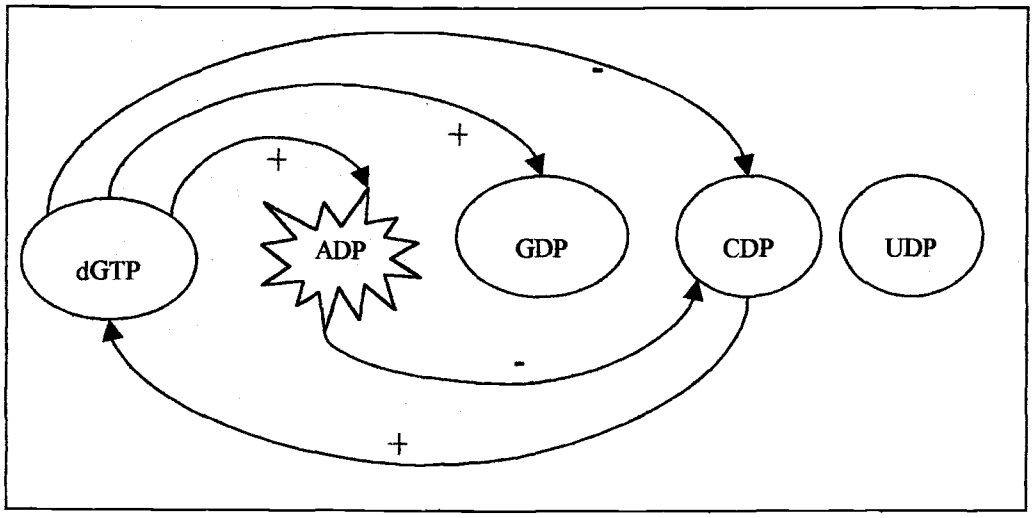


Model B dGTP Binding

1) Balanced rNDPs



2) Imbalanced rNDPs (High [ADP])



The two models can function in concert to maintain a well-balanced combination of rNDP substrates for RNR, which leads eventually to a proportional fraction of DNA precursors. Although they represent a potential way of regulating rNDP levels inside the cell, additional experiments are necessary for testing regulatory roles of other substrates in each case. However, it is conclusive that RNR is controlled by both allosteric effectors and substrates at their optimal concentrations.

Biological Significance of ADP Inhibition

The biological significance of ADP inhibition, other than to keep balanced pool sizes, is still unknown. An attempt to investigate whether the inhibitory effect of ADP upon its own reduction is to prevent a complete shutdown of DNA replication during low energy state of the cell did not confirm the hypothesis. The ADP/ATP ratios of V79 hamster lung cells have been successfully altered by the use of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). However, the data obtained from the enzymatic dNTP pool assays do not suggest that the inhibitory effect of ADP represents a link between energy metabolism and DNA synthesis for at least two reasons. First, there is no significant difference of the dATP pool levels between the untreated and FCCP-treated cells. Second, changes in the dNTP pools observed do not correspond to the altered ATP/ADP ratios. Whatever the biological role might be, it is certain that ADP plays an important

role in regulating the binding of dNTPs at the specificity site indirectly by displacing other substrates at the catalytic site. As a result, a balanced production of dNTPs can be maintained. Although it has been proven in Chapter 5 that ADP does not compete with dATP in binding to the high affinity site, the possibility that ADP mimics ATP and binds to the activity site cannot be ruled out. A recent study on a model for the allosteric regulation of mammalian RNR has shown that ATP can bind to a newly described site called hexamerization site (Kashlan *et al.*, 2002). Similarly, it is feasible that ADP is able to bind to a novel binding site.

Regulation of Mouse RNR Specificity by Hypoxic Environments

As illustrated in Chapter 6, the differential effects of oxygen limitation upon the substrate specificity are apparent. UDP reductase activity is the most resistant to hypoxic conditions, whereas GDP reductase activity is the most sensitive. The decrease in dGDP formation is the most pronounced at 1.5% O_2 (Figure 6.2). Therefore, these results do not agree with the hypothesis that the reduced O_2 content directly affects CDP reductase activity, leading to specific dCTP depletion. The basis for a specific decrease in dGDP formation still needs further investigation. However, two possibilities—a slow regeneration of GDP reductase activity and discrepancies in allosteric forms of the enzyme—have been ruled out. Perhaps the low level of dGDP production reflects natural dNTP pool asymmetries inside the cell. This is possible since it has been reported that the

dGTP pool in many types of mammalian cells is always underrepresented (5-10% of total amounts of dNTPs) (Mathews and Ji, 1992). Additionally, relative rates of dGDP formation by both *E. coli* and T4 bacteriophage RNRs, analyzed by the four-substrate assay consistently appear the lowest (Hendricks Ph.D. thesis, 1998). Hence, this phenomenon may be a common feature among several forms of Class I RNRs.

The anaerobic preincubation experiment (Figure 5.1) indicates that the oxygen-linked iron center is quite stable. Incubation of the enzyme under anaerobiosis for up to 60 minutes results in less than 50% activity loss, suggesting that the iron center is well retained and its turnover is relatively low once the catalysis starts. It is also implied that the tyrosyl free radical, once formed by the same process, needs not to be regenerated during repeated catalytic cycles. Our data agree with the concept of stability of the iron center once formed under aerobic conditions. The studies on kinetics of CDP reduction under anaerobic environments by mouse RNR previously exposed to O_2 support this idea. In fact, it has been demonstrated that the enzyme undergoes approximately 30 catalytic cycles before a complete activity loss (Thelander *et al.*, 1983).

Complex Regulation of Mouse RNR Specificity

Thus far, it has been discovered that the substrate specificity of mouse ribonucleotide reductase is controlled by several factors. First, the dNTP allosteric

modifiers—dATP, dGTP, and dTTP—play a major role in allosterically regulating the relative rates of dNDP product formation to maintain a balance of the intracellular dNTP pools. What makes this type of allosteric regulation more complicated is the fact that each dNTP affects the four activities differentially in a concentration-dependent manner. Therefore, the possibility that a gradient of dNTPs and compartmentation exist *in vivo* cannot be ignored. The four-substrate assay is a powerful tool that allows simultaneous monitoring of the four activities under conditions close to actual physiological environments; the reactions can be carried out in the presence of all four rNDP substrates and modifiers at manipulated concentrations. However, estimation of amounts of nucleotides present in the cell is a challenge, and thus, may not be absolutely accurate.

Our data have indicated that it is much more difficult now to predict the proportion of the dNDP products generated by the enzyme. This is partly due to the second factor—rNDP substrates. The turnover rates of substrates can be influenced by the rNDP substrates themselves as well as the dNTP allosteric modifiers. The competition among the substrates for the catalytic site has been demonstrated. Our results show that there is a two-way communication between the catalytic site and the specificity site. That is, binding of certain substrates such as CDP and GDP at the catalytic site directly affects the affinity of dNTP binding at the specificity site. ADP, in contrast, appears to have a distinct effect. It effectively competes with other substrates in binding to the catalytic site and removes their effects upon dNTP binding.

Oxygen limitation is the third factor that controls the enzyme specificity. Unexpectedly, differential effects upon the four activities were observed. Although it does not prove that dCTP deletion serves as a signal under anaerobiosis as a result of CDP reduction by RNR in response to hypoxia, the fact that GDP reductase activity is the most sensitive to low O_2 contents may explain, at least partly why dGTP is underrepresented inside the cell.

Recently, a new model for allosteric control of mouse RNR activity based on ligand binding, molecular mass and enzyme activity studies has been proposed (Kashlan *et al.*, 2002). This model suggests that oligomerization of the large subunits induced by nucleotide binding, especially ATP or dATP, determines the activity of the enzyme. For example, binding of ATP to the activity site and specificity site induces dimer formation of the R1 subunit ($R1_2$), whereas binding of dATP to these same sites leads to tetramer formation ($R1_4$). Interestingly, their data indicate a putative new binding site for ATP, so called an "h" site. Binding of ATP to this site results in hexamerization ($R1_6$). The enzyme activity studies show that $R1_2$ and $R1_6$ form an active form of the enzyme with $R2_2$. However, a complex formed between $R1_4$ and $R2_2$ displays only little activity. It is interesting that oligomerization also depends on concentrations of the nucleotides as well as the R1 protein.

Integrating these data to our discoveries suggest that the range of ATP and dATP concentrations used in the four-substrate assay leads to a mixture of tetramers and hexamers. At an ATP concentration of 2.5 mM, the hexamer form is

predominant. dATP at a concentration of 60 μM (estimated *in vivo* concentration) primarily induces formation of the tetramer form. Moreover, it has been reported that binding of dTTP, dGTP, UTP, GTP, ADP, and ddATP at higher concentrations induces at least partial tetramer formation. This means that configuration of the enzyme in our reaction mixture is not homogeneous. If this is the case under our assay conditions, different forms of the enzyme may, at least partly, contribute to differential effects of ligand binding upon the enzyme specificity. In addition, if ADP at high concentrations induces formation of $R1_4$, which when formed a complex with $R2_2$ leads to little enzyme activity, part of the inhibitory effect of ADP may be explained by this phenomenon. Hence, R1 oligomerization resulting from binding of different nucleotide ligands at various binding sites should also be taken into account. Considering all possible factors that can contribute to the total outcome of substrate specificity of the enzyme, it is not surprising that allosteric regulation of RNR explored up until now is, indeed, intriguing yet much more complex than that explained a few decades ago.

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Appendix

Mouse/Vaccinia Virus Chimeric Ribonucleotide Reductases

Due to the close structural relationship between ribonucleotide reductases of mouse and vaccinia virus (nearly 80% amino acid sequence identities (Tengelson *et al.*, 1988)), it was of our interest to test whether the two enzymes are also closely related functionally. It was shown for the first time by Hendricks and Mathews (1998a) that a hybrid enzyme containing vaccinia virus R1 (vvR1) and mouse R2 (mR2) was enzymatically active. This report then raised an interesting question of whether chimeric enzymes existed *in vivo* such as in vaccinia virus-infected cells. Here, studies on mouse-vaccinia virus RNR chimera have been followed up, using both hybrid enzymes (vvR1 + mR2 and mR1 + vvR2). It is confirmed that the chimeric RNR holoenzyme composed of mouse and vaccinia virus RNR subunits is functional. As illustrated in Figure I, consistent with the previous report, the vaccinia virus R1/mouse R2 (vvR1 + mR2) hybrid is active, although its specific activity is much lower than that of either native holoenzyme. The mouse R1/ vaccinia virus R2 (mR1 + vvR2) hybrid also shows some activity. However, surprisingly, a specific activity of this hybrid enzyme is significantly higher than that of either native enzyme. These data suggest that viral mutants lacking either R1 or R2 proteins may be able to survive by forming such chimeras with the host's RNR subunits in vaccinia virus-infected cells.

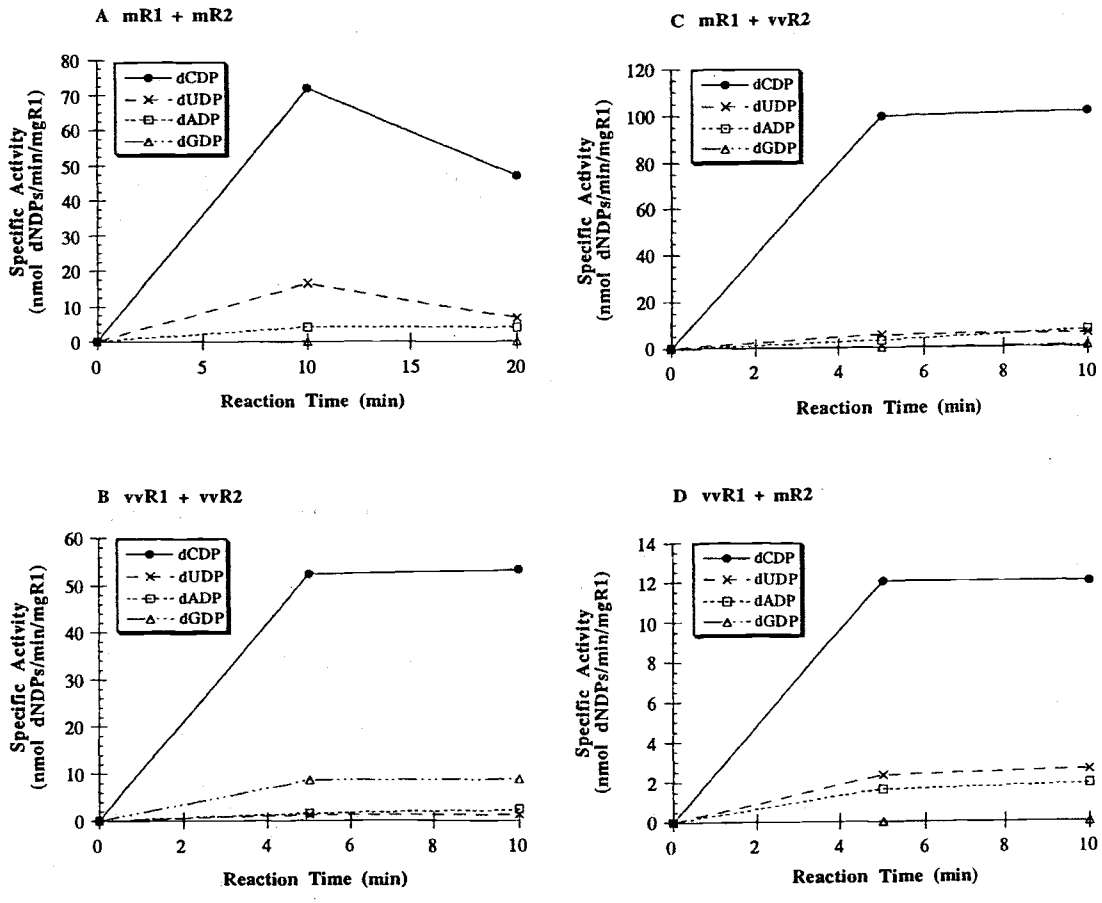

Figure I Activities of ribonucleotide reductase heterotetramers. The four-substrate assays were performed in the presence of all four rNDP substrates at 0.15 mM each and ATP at 2.5 mM as a sole allosteric effector. A, mouse RNR holoenzyme; B, vaccinia virus RNR holoenzyme; C, mouse R1 plus vaccinia virus R2; D, vaccinia virus R1 plus mouse R2.