Ribonucleotide Reductases: Amazing and Confusing

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Ribonucleotide reductases play a central role in DNA biosynthesis catalyzing the conversion of NTPs to dNDPs (dNTPs) (1–5) concomitant with oxidation of thiols within their active sites (Equation 1) (6). A single active site can accommodate reduction of both purines and pyrimidines. With the exception of the viral reductases (7) the substrate specificity of the proteins is governed by the choice of appropriate allosteric effectors (5, 8). The importance of maintaining controlled dNTP pool sizes to the fidelity of DNA replication mandates interactive multifaceted regulatory mechanisms of ribonucleotide reductases. These include transcriptional (9) and translational control (10) and perhaps enzymatic modulation of the production and destruction of active cofactor (11–13). The recent cloning, sequencing, and expression of reductases from a variety of sources (14–22) should allow a detailed investigation of these regulatory mechanisms in the next few years. The central role of ribonucleotide reductases in DNA biosynthesis also has made them a desirable target for the design of antitumor agents (5, 23, 24) and more recently antiviral agents (25–30).

EQUATION 1. TR, thioredoxin; TRR, thioredoxin reductase; (P)PP, pyrophosphate (tripolyphosphate).

Ribonucleotide reductases have been purified to homogeneity from a number of sources, and quite amazingly they differ substantially in structure (subunit size and organization) and in cofactor requirement (Table I). While at first glance the diversity of cofactor and subunit motifs seems irreconcilable, a common mechanistic theme emerges. Each of these proteins may provide an independent method for the generation of a protein radical and subsequently a substrate radical which could be directly involved in NDP (NTP) reduction. Extensive efforts in the past few years have been made utilizing isotopically labeled substrates and substrate analogs (mechanism-based inhibitors) and protein analogs (site-directed mutants) to provide support for the direct involvement of radical intermediates in ribonucleotide reductase-catalyzed reactions of enzymes from both Escherichia coli and Lactobacillus leichmannii. The evidence in support of these intermediates and their involvement in a unique mechanism of reduction is the focus of this review.

E. coli Ribonucleoside Diphosphate Reductase: Subunit Organization and Cofactor Structure

The ribonucleotide reductase from E. coli has been extensively investigated and is the prototype for the mammalian, yeast, and herpes simplex virus I and II proteins. The enzyme is composed of two subunits designated B1 (173 kDa) and B2 (87 kDa), each of which is composed of two equivalent protomers. The active site is proposed to be located at the interface of these subunits which are in a putative 1:1 complex (2). B1 and B2 have been cloned and overproduced separately (31–33). The turnover number of B1 is 200 min⁻¹ and that of B2 is 775 min⁻¹ when assayed in the presence of an excess of the second subunit. These differences in turnover numbers and the absence of any information concerning the Kₚ of the subunits indicate that subunit interactions and their stoichiometry during turnover require further investigation.

The cofactor required for E. coli ribonucleotide reductase activity resides on the B₂ subunit. Recent evidence using plasma emission spectroscopy to determine iron stoichiometry and Mossbauer spectroscopy to determine structure suggests that there is one binuclear iron center and tyrosyl radical/protemer (34). The largest number of tyrosyl radicals/B₂ reported to date, however, is 1.5 (34). These recent studies suggest a reevaluation of the dogma that has existed in the literature for the past decade that there is one binuclear iron center and one tyrosyl radical/B₂. Whether both protomers of B₂ are active and in fact what the specific activity of 100% active B₂ is has not yet been established.

Physical biochemical studies and chemical model systems have provided insight into the structure of the binuclear iron centers and tyrosyl radicals located on B₂ (35–38). The ions are high spin and in the 3+ oxidation state and are coupled to each other antiferromagnetically through a μ-oxo bridge. Extended x-ray absorption field spectroscopy data suggest that both ions are coordinated to N and O ligands (39, 40). NMR studies indicate that at least one of the ligands is a histidine (41). Similarities of the biophysical properties of B₂ to hemerythrin suggest that the iron rings are bridged via carboxylates from aspartates or glutamates. Comparison of gene sequences from five sources show approximately 20 conserved residues including 2 histidines, 3 aspartic acids, and 5 glutamic acids (42). These are potential candidates for iron ligands. The imminent x-ray structure of the B₂ subunit should unambiguously define the cofactor structure (43). Temperature-dependent EPR studies suggest that the tyrosyl...
The tyrosyl radical is characterized by a sharp absorbance at 410 nm (ε = 3900 M⁻¹ cm⁻¹) and a doublet EPR spectrum (g = 2.0047) with superposition of additional hyperfine interactions (45). Electron nuclear double resonance studies (46) and resonance Raman studies (47) have established that the tyrosyl radical is a phenoxy radical and is not a protonated (cation) radical as originally proposed. Sequence comparisons and site-directed mutagenesis studies have established that Tyr-122 is the oxidized residue (48). Reduction of the tyrosyl radical by hydroxurea or a number of one-electron reductants destroys the radical and all catalytic activity (49).

Recent efforts have focused on the mechanism of assembly of the binuclear iron center and tyrosyl radical of B1, both in vitro and in vivo. B2 with an intact iron center and a tyrosyl radical reduced by hydroxurea can be reassembled into an active center in the presence of a ferric reductase, Fe³⁺, or (an) unknown factor(s) and O₂. Superoxide dismutase is also required to prevent harmful production of O₂⁻ by reduced flavin oxidation (11–13). The ferric reductase presumably reduces both ferric ions of the binuclear center to ferrous which in the presence of O₂ and a reductant self-assemble into the active center. The best recovery of enzymatic activity via this method is 3.5 units mg⁻¹ compared with 8.5 units mg⁻¹ obtained from the best B2 preparation. These interesting observations require additional purification and reconstitution experiments to address mechanistic questions. Moreover, genetic dissection, such as deletion of the genes for the putative proteins involved in this process, is required to establish their physiological relevance. In vitro studies indicate that Fe³⁺, O₂, and a “reductant” are sufficient to form active cofactor from apoenzymes. Potential problems with Fenton’s chemistry have plagued interpretation of results attempting to determine the stoichiometry of Fe³⁺, O₂, and reductant required for maximal activity. In the mammalian system, reconstitution of the apo “M₂” requires only Fe³⁺, O₂, and DTT. Recent cloning and expression of the mammalian reductase should now facilitate a detailed investigation of this interesting process (15). Whether the iron center plays a role in addition to generating the tyrosyl radical remains to be established. At present no evidence exists for redox chemistry of the iron center during NDP reduction.

The B₁ subunit is the business end of ribonucleotide reductase. It binds the NDP substrates (both purines and pyrimidines) as well as the allosteric effectors which control in E. coli both the substrate specificity and turnover number (5). In mammalian systems, a similar complex regulatory cascade exists which appears to vary depending on the cell line and requires additional extensive investigation (8, 51). B₁ also contains a number of redox-active thiols which are oxidized concomitantly with NDP reduction (52). Recent studies designed to identify these thiols have suggested juxtaposition of at least five thiols in three-dimensional space (Cys-754, -759, -225, -230, and -462) (53–55). Depending on the method of alignment of gene sequences from seven sources, Cys-225, -439, and -462 appear to be conserved (5). Residues 754 and 759 are located in the C-terminal end of B₁. A sequence comparison of B₁, equivalents from a variety of sources reveals that they all possess two cysteines in their C-terminal ends with either a Cys-X-Cys or Cys-X-X-Cys-X-Cys motif. Similar motifs are frequently found in proteins involved in thiol/disulfide redox chemistry (1). The observation of multiple thiol/disulfide interchange range of one another led to the proposal that two thiols could be involved in the transfer of electrons between thioredoxin and the active site of B₁, i.e., an electron shuttle, while a second set of thiols could be directly involved in NDP reduction (55). The function of the thiols has recently been addressed using site-directed mutagenesis and will be discussed subsequently (53, 54, 56).

**Proposed Mechanism of Substrate Reduction**

The demonstration of an essential tyrosyl radical required for substrate reduction and the strong support for radical involvement in AdoCbl-requiring proteins in general has provided the impetus for a proposal of a mechanism for NDP reduction involving both protein radicals and substrate radicals. The working hypothesis shown in Scheme I is based on model chemistry previously discussed in detail (1, 4, 57). The basic principles to be tested are 1) 3' carbon-hydrogen bond cleavage mediated by a protein radical; 2) involvement of radical intermediates; and 3) reduction of a radical cation intermediate by thiols within the active site by one-electron transfers.

Studies using [3'-'H or -'H]NDPs established with E. coli ribonucleotide reductase that the 3' C–H bond is cleaved and that H abstracted from the 3' position in the starting material is returned to the same position in the product (58). Efforts to establish direct involvement of the tyrosyl radical in mediating 3' hydrogen atom abstraction using stopped flow kinetics under a wide variety of conditions have failed (1, 4). Efforts to establish intermediacy of radicals and correlation of protein radical chemistry with 3' C–H bond cleavage of substrate have therefore focused on two alternative approaches to change rate-determining steps: 1) use of mechanism-based inhibitors, altered substrates (23, 59–63); and 2) use of site-directed mutagenesis, altered proteins (53, 54).

**Altered Substrates: Mechanism-based Inhibitors**

Two mechanism-based inhibitors, 2'-chloro-2'-deoxyuridine 5'-diphosphate (CI-UDP) (23, 59, 60) and 2'-azido-2'-deoxyuridine 5'-diphosphate (M₀ UDP), have therefore focused on two alternative approaches to change rate-determining steps: 1) use of mechanism-based inhibitors, altered substrates (23, 59–63); and 2) use of site-directed mutagenesis, altered proteins (53, 54).

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* M. J. Bollinger and J. Stuthe, unpublished results.
* The abbreviations used are: DTT, dithiothreitol; AdoCbl, adenosylcobalamin; CI-UDP, 2'-chloro-2'-deoxyuridine 5'-diphosphate; N₃UDP, 2'-azido-2'-deoxyuridine 5'-diphosphate; M₀, the mammalian counterpart to B₁.
deoxyuridine 5'-diphosphate (N3UDP) (23, 61-63), have played a critical role in defining the catalytic capabilities of reductases. Extensive studies on the interaction of CI-UDP with ribonucleoside diphosphate reductase have revealed the following scenario. CI-UDP is converted to 3'-keto-UDP via a 1,2-H shift which can collapse to produce the highly reactive furanone leading to enzyme inactivation (Equation 2). While the 3'-C-H bond of CI-UDP is cleaved during this reaction, all efforts using stopped flow kinetics to correlate 3'-C-H bond cleavage and tyrosyl radical reduction have failed. Model studies provide an explanation for the differences between the reaction of CI-UDP and UDP with ribonucleotide reductases and have been discussed in detail elsewhere (59, 60).

**Equation 2.**

Interaction of N3UDP with ribonucleoside diphosphate reductase has also been investigated in great detail (61-65). These studies are the first to reveal the ability of ribonucleotide reductase to mediate radical-dependent chemistry (Equation 3). One equivalent of N3UDP totally inactivates the enzyme, producing 1 eq of uracil, N3, PP, and "sugar" label bound specifically to B1. No N3 is released. Greater than 90% of the tyrosyl radical is destroyed (biphasic kinetics), and a new nitrogen-centered radical derived from the N3 moiety of N3UDP is formed. The biphasic kinetics observed on examination of the rates of inactivation and loss of the tyrosyl radical of B2 may in fact be related to the inability of both protomers of B2 to catalyze the observed chemistry. The B2 subunit might have to dissociate and reassociate in a different configuration with B1, and this could be the second slow phase of the reaction (61, 62). Studies using 2'-3C-15N-labeled N3UDP suggest that the nitrogen-centered radical is no longer attached to the sugar ring. The observation of an absorbance change at 320 nm on the protein accompanying enzyme inactivation is also reminiscent of the furanone formation observed with CI-UDP and is consistent with "N" of the azide moiety no longer being attached to C-2'. Recent studies of N3UDP with site-directed mutants of B1 in which cysteine 225 is replaced with a serine (Cys-225 + Ser) or cysteine 462 (Cys-439 + Ala) mutant. Incubation of this mutant with CDP in the presence of DTT as reductant also produces cytosine (1), reminiscent of results observed with both mechanism-based inhibitors N3UDP and CI-UDP (53, 54). The need to establish product identification is reemphasized by recent studies with Cys-462 + Ser and Cys-759 + Ser mutants (53, 54). These studies were also plagued with the problem of contaminating wild type enzyme, but with the Cys-225 + Ser mutant an amazing sequence of events was observed during its incubation with its normal substrate CDP (Equation 4).

**Equation 4.**

To further define if the tyrosyl radical is directly involved in catalysis and to define which thiols are involved in substrate reduction, site-directed mutagenesis studies have been undertaken (53, 54, 56). The rationale in the absence of structural information was that alteration of the redox thiols would prevent reduction and perhaps allow measurement via stopped flow kinetics of a correlation between cleavage of the 3'-C-H bond and destruction of a tyrosyl radical (53, 54). While alanine and serine site-directed mutants and double mutants of cysteines 225, 439, 462, 754, and 769 have been produced, unambiguous interpretation of data under normal assay conditions is severely hampered by an undeterminable amount of contaminating wild type B1. In one set of experiments (Cys-X → Ala) mutants of cysteines 225, 230, 754, 759, 439, and 462 were prepared and assayed under multiple turnover conditions with a variety of reductants (56). The conclusion was reached based on low "turnover" of Cys-225 → Ala and Cys-439 → Ala (4-6% of wild type) that these thiols were in the active site. Since Cys-759 → Ala and Cys-754 → Ala appeared to be active with DTT as reductant and possessed low activity (3% wild type) with thioredoxin/thioredoxin reductase as the reductant, a second conclusion was reached that these thiols functioned as the redox shuttle between B1 and thioredoxin (56). Unfortunately in addition to the problem of contamination with an unknown amount of wild type B1, the products of the reaction were not analyzed (56). Analysis of the products produced is absolutely required for unambiguous interpretation of results based on earlier studies with Cys-225 → Ser and Cys-759 → Ser mutants (53, 54). These studies were also plagued with the problem of contaminating wild type enzyme, but with the Cys-225 → Ser mutant an amazing sequence of events was observed during its incubation with its normal substrate CDP (Equation 4).

The major product formed by the interaction of Cys-225 + Ser with CDP is not dCDP but is cytosine (1), reminiscent of results observed with both mechanism-based inhibitors N3UDP and CI-UDP (53, 54). The need to establish product identification is reemphasized by recent studies with Cys-462 + Ser mutant. Incubation of this mutant with CDP in the presence of DTT as reductant also produces cytosine! Incubation with thioredoxin/thioredoxin reductase as reductant results in rapid inactivation of the contaminating wild type enzyme and presumably the mutant enzyme. Inactivation is accompanied by a change in absorbance of the protein at 320 nm and is probably the result of alkylation of the protein by the furanone produced when base is released from a 3'-ketoexonucleotide. When DTT is utilized as reductant, it reacts with the furanone and prevents enzyme inactivation. These results are strikingly similar to those previously observed with CI-UDP (59, 60).

While a tremendous effort is required to unravel the complexity of the role of thiols, it is clear that a detailed investigation of the mutant ribonucleotide reductase interactions with NDP, CI-NDP, and N3NDP will provide much insight into the catalytic capabilities of these enzymes. It also appears...
that five thiols are involved in catalysis, Cys-754 and -759 as a redox shuttle (51, 56) and Cys 225, -462, and -439 (53, 54, 56) within the active site. Recent x-ray data suggests that the tyrosyl radical is buried within B2 making it unlikely that it could function as the putative X' in Scheme I (65). Is it possible that long range electron transfer occurs between it and another residue, perhaps Cys-439 on the B1 subunit to mediate the chemistry? This speculative proposal suggests that the sole function of B2 is to generate a protein radical on B1. Residues 225 and 462, both of which convert the substrate into a mechanism-based inhibitor and previously identified as active site redox thiols by isoelectrofocusing labeling experiments, might be directly involved in reduction (55). While highly speculative, the thesis is experimentally testable. As will be outlined below, a similar thiol-mediated mechanism can be proposed for L. leichmannii reductase which although structurally distinct from the E. coli enzyme catalyzes remarkably similar chemistry (1, 4).

**L. leichmannii Reductase**

The ribonucleotide reductase from *L. leichmannii* is a single polypeptide of M, = 76,000 and utilizes AdoCbl as a cofactor (turnover is 115 min^{-1}). The *Lactobacillus* enzyme serves as a prototype for a variety of other B12-requiring reductases (1, 3, 66). Ribonucleoside triphosphosphate reductase catalyzes the reduction of NTPs to dNTPs concomitant with oxidation of thiols (67). *E. coli* thioredoxin can function to recycle the oxidized *Lactobacillus* enzyme. In addition ribonucleoside triphosphate reductase also catalyzes an unusual exchange reaction (68, 69). Incubation of [5'-3H]AdoCbl with ribonucleoside triphosphate reductase results in production of 3H2O in a process which requires both reductant and an allostERIC effector. The reaction is intriguing even though 3H release is slow (10% the rate of substrate reduction) and is not observed during the normal reduction of NTP (68-70). The observation of 3H2O production and rapid C O bond homolysis requires the involvement of a second radical. A protein radical provides the most reasonable model (Equation 5). The linkage between this exchange reaction and the normal reduction process will be addressed subsequently.

![Equation 5](image)

A mechanism for reduction of NTP to dNTP identical to that proposed for the *E. coli* reductase (Scheme I) is also appropriate for that catalyzed by ribonucleoside triphosphate reductase (71). Studies using [3'-3H]-H and -H]NTPs have established as in the case of the *E. coli* enzyme that the 3' C-H bond is cleaved and that the hydrogen abstracted from the 3' position is returned to the 3' position in the product (71). Previous studies have shown that ribonucleoside triphosphate reductase catalyzes homolytic cleavage of the carbon- cobalt bond to produce cobelamin(II) and 5' deoxyadenosyl radical in a kinetically competitive fashion (72, 73). One turnover experiments using [3'-3H]UTP ([3'-3H]ATP have established, however, that the 5'-deoxyadenosyl radical is not directly involved in H closure from the substrate (67)!

Thus X' in Fig. 1 is not the 5'-deoxyadenosyl radical. The results are consistent with the 5'-deoxyadenosyl radical generating a protein radical X' capable of mediating the chemistry. Thus the X' mediating the exchange reaction (Equation 5) may be the same X' involved in substrate reduction. These results in sum are remarkably similar to those observed with *E. coli* ribonucleoside diphosphate reductase.

Amazingly enough, interaction of the mechanism-based inhibitor Cl-UTP with ribonucleoside triphosphate reductase results in rapid enzyme inactivation accompanied by products identical to those described in Equation 2 for the *E. coli* reductase (59, 74). Furthermore, studies attempting to identify the active site thiols using ATP and one turnover conditions and residue(s) modified by the Cl-UTP mechanism-based inhibitor revealed three thiols and two peptides (55). One peptide has sequence homology remarkably similar to the C-terminal peptide of the *E. coli* reductase (Scheme II), perhaps the sequence recognized by thioredoxin. Could the third thiol be X' in Scheme I? Could X' be generated by long range e-transfer mediated by the 5'-deoxyadenosyl radical? Efforts are underway to establish the nature of X' in this and the *E. coli* reductase.

**E. coli: GCGGACTK**

**L. leichmannii: -CGGACP1K**

**SCHEME II**

**Other Reductases**

Recently ribonucleotide reductase has been isolated from *Brevibacterium ammoniagenes* and has been shown to be composed of an αβ subunit structure α (80 kDa) and β (100 kDa) (75). The cofactor contains "manganese," and spectroscopic data in comparison with chemical model systems suggests the existence of a novel binuclear manganese center analogous to the binuclear iron center in ribonucleotide reductase from *E. coli* (35, 36). Sensitivity of this enzyme to inactivation by hydroxyurea also suggests involvement of a tyrosyl radical. Within the past year a fourth class of reductase has been isolated from *E. coli* grown under anaerobic conditions (76). Neither the subunit structure nor the cofactor has yet been unambiguously established. Based upon studies with pyruvate formate lyase, an enzyme requiring an anaerobic mechanism for generation of a protein radical required for activity, however, one might speculate that the cofactor of anaerobic *E. coli* ribonucleotide reductase might be iron and S-adenosylmethionine (64, 76).

While the structures of cofactors involved in the manganese reductases and anaerobic *E. coli* reductases remain to be established, one thing is for certain, the chemistry involved will be novel. As outlined briefly above, both the chemistry and biology of reductases are very complex. With the tools presently at hand, the next few years are certain to unveil many of the mysteries which still surround these key enzymes involved in DNA biosynthesis.

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