

Coenzyme B₁₂-Dependent Ribonucleotide Reductase: Evidence for the Participation of Five Cysteine Residues in Ribonucleotide Reduction[†]

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ABSTRACT: Ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* catalyzes the conversion of ribonucleotides to 2'-deoxyribonucleotides and requires adenosylcobalamin (AdoCbl) as a cofactor. Recent cloning, sequencing, and expression of this protein [Booker, S., & Stubbe, J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 8352–8356] have now allowed its characterization by site-directed mutagenesis. The present study focuses on the role of five cysteines postulated to be required for catalysis. The choice of which of the ten cysteines of RTPR were to be mutated was based on extensive studies on the *Escherichia coli* ribonucleoside diphosphate reductase. Despite the differences between these two reductases in primary sequence, quaternary structure, and cofactor requirements, their mechanisms are strikingly similar. The mutagenesis studies reported herein further suggest that the complex role of the five cysteines is also very similar. A variety of single and double mutants of RTPR were prepared (C731S, C736S, C731 and 736S, C119S, C419S, C408S, and C305S), and their interaction with the normal substrate (CTP) was characterized under several sets of conditions. Mutants C731S, C736S, and C731 and 736S all catalyzed the formation of dCTP at rates similar to those of the wild-type (wt) enzyme in the presence of the artificial reductant DTT. In the presence of the *in vivo* reducing system (thioredoxin, thioredoxin reductase, and NADPH), however, each of these mutants catalyzed the formation of only 0.6–0.8 dCTPs per mole of enzyme. The inability of these mutants to catalyze multiple turnovers with respect to the *in vivo* reducing system suggests that their function might be to transfer reducing equivalents from thioredoxin into the active site disulfide of the reductase. Mutants C119S and C419S were targeted as being the active site cysteines, the ones which directly reduce the ribonucleotide substrate. As expected, neither of these mutants catalyzed the formation of dCTP. However, they did catalyze a time-dependent formation of cytosine, destruction of the cofactor, and the appearance of a chromophore associated with the protein—all phenotypes previously observed for the corresponding active site cysteines of the *E. coli* reductase. Mutant C408S was unable to catalyze dNTP production or cytosine release. Moreover, it was ineffective in catalyzing two additional reactions which are unique to this enzyme: the exchange of tritium from the 5' hydrogens of AdoCbl with H₂O and the destruction of AdoCbl under anaerobic conditions to give 5'-deoxyadenosine and cob(II)alamin. These results are consistent with the role of this cysteine as the protein radical responsible for initiating catalysis.

The ribonucleotide reductases constitute a unique class of metalloenzymes which catalyze the reduction, at a single active site, of both purine and pyrimidine ribonucleotides to the corresponding 2'-deoxyribonucleotides (dNTPs).¹ Because the reductases provide the cell its only means of making the dNTPs necessary for DNA biosynthesis, these enzymes are essential components of biological systems as simple as the T4 phage and herpes simplex viruses and as complex as humans

and other mammals (Eriksson & Sjöberg, 1989; Stubbe, 1990a; Reichard, 1993a). Their uniqueness is particularly intriguing in that, in contrast to most other enzymes that play key roles in primary metabolism, the reductases are not conserved with respect to quaternary structure and cofactor requirement.

The ribonucleotide reductases have been classified on the basis of their cofactor requirement and quaternary structure. Class I reductases are represented by the ribonucleoside diphosphate reductase (RDPR) isolated from *Escherichia coli*. Enzymes in this class are tetramers constructed of two homodimeric proteins, R1 and R2. The smaller protein, R2, houses a diferric iron center–tyrosyl radical cofactor which is absolutely necessary for substrate turnover. Other reductases in this class are from mammalian systems, as well as the herpes simplex viruses (Averett et al., 1983; Dutia, 1986; Stubbe, 1990b; Reichard, 1993a). Class II enzymes are structurally the simplest of all the reductases and are represented by the ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* (Blakely, 1978; Lammers & Follmann, 1983). RTPR functions as a single polypeptide of 82 kDa (Booker & Stubbe, 1993) and requires coenzyme B₁₂ (AdoCbl) for dNTP production. The enzyme isolated from *Brevibacterium ammoniagenes* is the prototype for class III reductases. This enzyme has an absolute requirement for manganese and is composed of two proteins,

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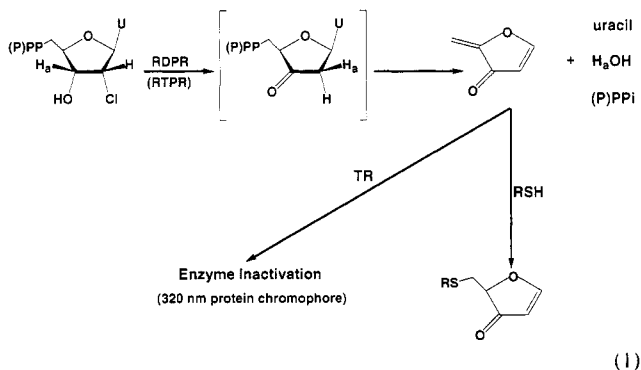
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¹ Abbreviations: AdoCbl, coenzyme B₁₂; B₁₂r, cob(II)alamin; CDP, cytidine 5'-diphosphate; CIP, alkaline phosphatase from calf intestine; CIUD(T)P, 2'-chloro-2'-deoxyuridine 5'-di(tri)phosphate; CTP, cytidine 5'-triphosphate; 5'-dA, 5'-deoxyadenosine; dATP, 2'-deoxyadenosine 5'-triphosphate; dC, 2'-deoxycytidine; dCTP, 2'-deoxycytidine 5'-triphosphate; dGTP, 2'-deoxyguanosine 5'-triphosphate; dNTP, 2'-deoxynucleoside 5'-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; LB, Luria-Bertani medium; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NTP, nucleoside 5'-triphosphate; PCR, polymerase chain reaction; PPP_i, triphosphosphate; RDPR, ribonucleoside diphosphate reductase; RTPR, ribonucleoside triphosphate reductase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TR, thioredoxin; TRR, thioredoxin reductase.

the smaller of which is a dimer and the larger a monomer (Willing et al., 1988a,b). Recently, a ribonucleotide reductase from *E. coli* grown under anaerobic conditions has been isolated. This class IV enzyme is a dimer and is proposed to use a [4Fe-4S] cluster in combination with *S*-adenosylmethionine and other small molecules to carry out substrate reduction (Eliasson et al., 1992; Reichard, 1993a,b).

Despite the differences in quaternary structure, cofactor requirement, as well as primary sequence, evidence suggests that at least two reductases, those from *Lactobacillus leichmannii* and *E. coli* grown under aerobic conditions, operate by very similar mechanisms of catalysis. First, both enzymes contain cysteine residues which become oxidized to a cystine concomitant with each substrate turnover event (Vitols et al., 1967a; Thelander, 1974). In both systems the active disulfide can be rereduced by an *in vivo* reductant, thioredoxin, or by small organic dithiols such as dithiothreitol (DTT), a requirement for multiple turnovers. Second, both enzymes initiate the catalytic event by a protein radical-mediated hydrogen atom abstraction from the 3' carbon of the nucleotide substrate (Stubbe et al., 1981, 1983). This hydrogen atom is subsequently returned to the same position in the deoxynucleotide product. In each system a protein radical is postulated to be generated by the metallocofactor (Stubbe, 1990b). Third, both enzymes display remarkably similar phenotypes when presented with 2'-halogenated 2'-deoxynucleotides. Studies with the mechanism-based inhibitor 2'-chloro-2'-deoxyuridine di(tri)phosphate [CIUD(T)P] revealed that, subsequent to 3' C-H bond cleavage, each enzyme catalyzed the formation of 3'-keto-2'-deoxyuridine 5'-di(tri)phosphate in which the hydrogen originally at the 3' carbon of the sugar is returned stereospecifically to the β face of the 2' carbon of the sugar or is transferred to the solvent (eq 1).



This intermediate then collapses to liberate free base (uracil), pyrophosphate (tripolyphosphate), and a 2-methylene-3(2H)-furanone which in each case alkylates the protein, yielding a chromophore at 320 nm (Harris et al., 1984; Lin et al., 1987; Ashley et al., 1988).

The mechanism of ribonucleotide reduction has also been investigated with the aid of protein analogs. Recent site-directed mutagenesis studies on the RDPR from *E. coli* have allowed a model to be proposed in which five cysteine residues act in concert to carry out the reduction process (Aberg et al., 1989; Mao et al., 1989, 1992a-c). All of these catalytically important cysteines are located on the R1 subunit of the enzyme, which is the subunit that binds the substrates as well as the allosteric effectors. Two C-terminal cysteines (754 and 759) function to deliver reducing equivalents from thioredoxin into the active site disulfide of RDPR (formed concomitant with substrate reduction), thus regenerating active enzyme. The active site cysteines (225 and 462) function to directly reduce the nucleotide substrate. Lastly, a fifth cysteine

is proposed to be converted into a thiyl radical via long-range electron transfer to the diferric iron center-tyrosyl radical cofactor on the second subunit, R2. This thiyl radical is then proposed to initiate catalysis by abstracting the 3' hydrogen atom of the substrate. While this model for the role of multiple cysteines in catalysis by the *E. coli* reductase is moderately compelling, the interpretation of the results was blemished by the presence of contaminating wild-type (wt) R1 in all of the mutants examined (Aberg et al., 1989; Mao et al., 1992a-c). The recent cloning, sequencing, and expression of the enzyme from *L. leichmannii* (Booker & Stubbe, 1993) have now made similar studies possible on a different class of reductase. Furthermore, our ability to express the *L. leichmannii* reductase in *E. coli* has eliminated the problem of contaminating wt in the mutants generated. The results of the studies of the interactions of five C \rightarrow S site-directed mutants of RTPR with CTP are presented. The phenotypes of these mutants are strikingly similar to the corresponding *E. coli* RDPR mutants, providing convincing evidence in conjunction with the mechanistic studies that the active sites of these enzymes are structurally similar.

MATERIALS AND METHODS

Dithiothreitol was purchased from Mallinckrodt. [^{14}C]-Cytidine 5'-diphosphate (CDP; 2.0 GBq/mmol), [$1',2',3\text{H}$]-deoxyguanosine 5'-triphosphate (dGTP; 1.3 TBq/mmol), and [$\gamma\text{-}^{32}\text{P}$]ATP (222 TBq/mmol) were purchased from New England Nuclear. Coenzyme B₁₂ (AdoCbl), 5'-deoxyadenosine (5'-dA), cytidine 5'-triphosphate (CTP), deoxyadenosine 5'-triphosphate (dATP), cytosine, 2'-deoxycytidine (dC), and NADPH were purchased from Sigma. Alkaline phosphatase (specific activity 3143 units/mg) from calf intestine (CIP) was purchased from Boehringer Mannheim. Restriction endonucleases were purchased from New England Biolabs. AmpliTaq DNA polymerase was from Perkin-Elmer/Cetus, and ultrapure dNTPs were from Pharmacia. T4 DNA ligase was purchased from GIBCO/BRL. Centricons and membranes for Amicon ultrafiltration devices were obtained from Amicon. Anion-exchange resin AG1-X2 (50-100 mesh) was purchased from Bio-Rad. [$5\text{'-}^3\text{H}$]AdoCbl (1×10^7 cpm/ μmol) was a generous gift from Professor H. P. C. Hogenkamp of the University of Minnesota (Minneapolis).

UV-visible absorption spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer. All scintillation counting was performed on a Packard 1500 liquid scintillation analyzer using 8 mL of S&INT-A XF scintillation cocktail (Packard) per 1 mL of aqueous reaction. High-pressure liquid chromatography (HPLC) was carried out using a Beckman 110 solvent delivery module, a 421A controller, and a 163 variable wavelength detector, in combination with an Alltech Econosil C₁₈ column. SDS-PAGE was performed as described by Laemmli (1970).

Wild-type *L. leichmannii* RTPR (specific activity 1.5 units/mg) was isolated from the *E. coli* overproducing strain pSQUIRE/HB101 (Booker & Stubbe, 1993). *E. coli* thioredoxin (TR) (specific activity 50 units/mg) and thioredoxin reductase (TRR) (specific activity 800 units/mg) were isolated from overproducing strains SK3981 (Lunn et al., 1984) and K91/pMR14 (Russel & Model, 1985). *E. coli* strain JM101 was obtained from Pharmacia. Chromosomal DNA from *L. leichmannii* (ATCC 7830) was isolated as described previously (Booker & Stubbe, 1993). Oligonucleotide primers used for DNA sequencing, mutagenesis, and the polymerase chain reaction (PCR) were obtained from the MIT Biopolymers Laboratory or Oligos Etc., Wilsonville, OR.

Preparation of [2-¹⁴C]CTP. [2-¹⁴C]CTP was prepared from [2-¹⁴C]CDP using the enzymes pyruvate kinase and lactate dehydrogenase. It was subsequently purified on a DEAE-Sephadex A25 column as described by Stubbe and Ackles (1980).

Preparation of Site-Directed Mutants. All of the mutant RTPRs except C305S were constructed by the polymerase chain reaction methods using standard procedures (Nelson & Long, 1989; Ho et al., 1989). Mutant C305S was made with the oligonucleotide-directed in vitro mutagenesis system (version 2.1) from Amersham. The details of the procedures as well as the primers used for mutagenesis are provided in the supplementary material. The integrity of each mutant protein was verified using the dideoxy chain-termination method of Sanger et al. (1977) in combination with the dsDNA cycle sequencing system from GIBCO/BRL.

Growth and Expression of Mutants. Plasmid pSQUIRE containing the appropriate mutant was transformed into *E. coli* JM101 and grown to saturation (10–12 h) in Luria broth supplemented with ampicillin (50 µg/mL). No isopropyl thio-β-D-galactoside was needed to induce the expression of RTPR. The mutant RTPRs were isolated by procedures identical to that which was previously described for wt RTPR (Booker & Stubbe, 1993) except that care was taken to ensure that the column resins had never previously been used to isolate the wt protein.

Enzyme Assays Using [2-¹⁴C]CTP and NaOAc. RTPR or the appropriate mutant was exchanged into 2 mM HEPES (pH 7.5) using a Sephadex G-50 column, and its concentration was determined by UV absorption ($E_{1\%}^{1\text{cm}} = 13.3$ at 280 nm) (Blakley, 1978). A typical assay contained in a final volume of 510 µL 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 M NaOAc, 30 mM DTT, 1 mM [2-¹⁴C]CTP (specific activity 7.5×10^5 – 1.5×10^6 cpm/µmol), 8 µM AdoCbl, and 0.10–0.12 nmol of wt or mutant RTPR. Alternatively, DTT was replaced with TR (108 µM), TRR (0.5 µM), and NADPH (2 mM). The reaction mixture was incubated for 5 min at 37 °C, and an aliquot (100 µL) containing everything except AdoCbl was removed at the 0 time point. All assays were carried out in the dark under dim red light, and at various times subsequent to the addition of AdoCbl, a 100-µL aliquot was removed and quenched in 200 µL of 2% perchloric acid. The contents were neutralized with 180 µL of 0.2 N NaOH and 50 µL of 0.5 M Tris-HCl, pH 8.5/1 mM EDTA. Subsequent to the addition of 10 units of CIP, the reaction was incubated at 37 °C for 1.5 h. To each reaction vial was added 30 µL of carrier cytosine and dC (120 nmol), and the reaction was diluted to ~1.5 mL with H₂O. Each reaction was loaded onto 0.75 × 7 cm AG1-X2 columns (borate form, 50–100 mesh) prepared by the method of Steeper and Steuart (1970). Each column was washed with 12 mL of H₂O, and a 1-mL portion of the eluate was subjected to scintillation counting. A 10-mL aliquot of the remaining 11 mL was concentrated to ~1 mL for reverse-phase HPLC analysis using an Econosil C₁₈ column with H₂O as the eluate. The column was washed with H₂O at a flow rate of 1 mL/min, and 1-mL fractions were collected and analyzed by scintillation counting. Cytosine and dC eluted isocratically in H₂O at ~7 and 20 min, respectively.

Enzyme Assays Using [2-¹⁴C]CTP and Allosteric Effector. Enzyme reaction mixtures were identical to that described above except that NaOAc was replaced with 0.12 mM dATP and 1 mM MgCl₂. Subsequent to the addition of AdoCbl to initiate the reaction, 100-µL aliquots were removed at various time intervals and quenched in 50 µL of 2% perchloric acid.

The contents were neutralized with 45 µL of 0.2 N NaOH and 20 µL of 0.5 M Tris-HCl, pH 8.5/1 mM EDTA. CIP (5 units) was added, and the reaction mixture was incubated at 37 °C for 1.5 h and then analyzed for cytosine and dC formation as described above.

Single-Turnover Experiments with Mutants C731S, C736S, and C731 and 736S. Wt RTPR or the appropriate mutant (37 nmol) was prereduced (in a total volume of 200 µL) for 20 min at 37 °C with 30 mM DTT in 50 mM HEPES (pH 7.5). This mixture was loaded onto a Sephadex G-50 column (0.75 × 7 cm) equilibrated in 2 mM HEPES (pH 7.5) to remove the DTT. Fractions containing protein ($A_{280\text{nm}}$) were pooled and concentrated. A 50-µL aliquot (9–12 nmol) was added to the reaction mixture which contained in a final volume of 510 µL 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 mM [2-¹⁴C]CTP (specific activity 1.4×10^6 cpm/µmol), 0.12 mM dATP, 1 mM MgCl₂, and 50 µM AdoCbl. Alternatively, 1 M NaOAc replaced the dATP and MgCl₂. The reaction mixture was incubated for 5 min at 37 °C, and an aliquot (100 µL) containing everything except AdoCbl was removed at the 0 time point. Subsequent to the addition of AdoCbl, 100-µL aliquots were removed at 1, 3, 10, and 20 min, quenched, and analyzed as described above.

Characterization of Oxidized RTPR. Prereduced RTPR (29 nmol) was added to a solution containing in a final volume of 200 µL 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 mM CTP, 50 µM AdoCbl, and 1 M NaOAc, and the solution was preincubated for 2 min at 37 °C. The reaction was initiated with AdoCbl and incubated for 3 min at 37 °C. It was then loaded onto a Sephadex G-50 column (0.75 × 7 cm) equilibrated in 2 mM HEPES (pH 7.5). The protein-containing fractions were pooled and concentrated to ~200 µL in a Centricon 30 ultrafiltration device. A 150-µL aliquot (17 nmol) of this enzyme was added to the single-turnover assay solution described above containing 1 mM [2-¹⁴C]CTP (specific activity 1.4×10^6 cpm/µmol) and 1 M NaOAc. Subsequent to the removal of a 100-µL aliquot ($t = 0$ min), the assay was initiated by the addition of AdoCbl. Additional 100-µL aliquots were removed at 10, 20, 30, and 40 min, quenched, and worked up as described above.

Determination of Product Production with Mutants C119S and C419S. The assay contained in a final volume of 250 µL 25 mM HEPES (pH 7.5), 1 M NaOAc, 4 mM EDTA, 1 mM [2-¹⁴C]CTP (specific activity 1.2×10^6 cpm/µmol), 20 µM TR, 0.12 µM TRR, 0.2 mM NADPH, 2.5 nmol of C419S or C119S, and 80 µM AdoCbl. Alternatively, DTT (30 mM) replaced the TR/TRR/NADPH reducing system, and/or dATP (0.12 mM) and MgCl₂ (1 mM) were substituted for NaOAc. The reaction mixture was incubated for 5 min at 37 °C, and an aliquot containing everything except AdoCbl, which was used to initiate the reaction, was removed at the 0 time point. After a 30-min incubation at 37 °C for C119S or a 60-min incubation for C419S, another 100-µL aliquot was removed, quenched, and worked up as already described depending on whether the assay was conducted with NaOAc or with dATP and MgCl₂.

Analysis of the Ability of C408S To Catalyze Nucleotide Reduction. The assay solution contained in a final volume of 310 µL 50 mM HEPES (pH 7.5), 4 mM EDTA, 50 µM AdoCbl, 1 mM [2-¹⁴C]CTP (specific activity 1.5×10^6 cpm/µmol), 24 µM C408S RTPR, 0.12 mM dATP, 1 mM MgCl₂, 108 µM TR, 0.5 µM TRR, and 2 mM NADPH. Alternatively, dATP and MgCl₂ were replaced with 1 M NaOAc, and/or TR/TRR/NADPH was replaced with 30 mM DTT. Subsequent to a 5-min preincubation at 37 °C, a 100-µL aliquot

was withdrawn ($t = 0$), and the reaction was initiated with the addition of AdoCbl. Additional 100- μ L aliquots were removed at 10 and 30 min and quenched for 2 min in a boiling water bath. Cytosine and dC production was analyzed by HPLC as described above.

Analysis of the Ability of C408S To Catalyze Exchange of $[5'\text{-}^3\text{H}_2]\text{AdoCbl}$ with H_2O . The assay solution contained in a final volume of 210 μ L 50 mM HEPES (pH 7.5), 0.3 mM dGTP, 4 mM EDTA, 65 μ M TR, 0.21 μ M TRR, 200 μ M $[5'\text{-}^3\text{H}_2]\text{AdoCbl}$ (specific activity 7.5×10^5 cpm/ μ mol), and 12 nmol of mutant C408S. All reaction components except dGTP were preincubated for 5 min at 37 °C. A 100- μ L aliquot (used as the control) was removed and incubated at 37 °C for the duration of the assay. dGTP was added to initiate the reaction, and subsequent to a 30-min incubation at 37 °C, a 100- μ L aliquot was removed, placed in a 10-mL pear-shaped flask, and shell-frozen in dry ice-acetone. The control reaction was treated in the same fashion, and both reactions were analyzed by bulb-to-bulb distillations to separate the volatile tritium from $[5'\text{-}^3\text{H}]\text{AdoCbl}$. The distillate containing $^3\text{H}_2\text{O}$ and the residue containing $[5'\text{-}^3\text{H}]\text{AdoCbl}$ were brought to a final volume of 1 mL with H_2O and analyzed by scintillation counting. Wt RTPR (0.12 nmol) was treated in the same fashion; however, the incubation time was reduced to 10 min.

RTPR-Catalyzed Cob(II)alamin Formation. The reaction mixture included in a final volume of 600 μ L 200 mM HEPES (pH 7.5), 100 μ M AdoCbl, 0.12 mM TR, 1 μ M TRR, 1 mM NADPH, and 120–150 μ M wt or C408S RTPR. This mixture was placed in a 10-mL pear-shaped flask fitted with a septum and purged with argon for 20 min at 4 °C with gentle stirring. A 450- μ L aliquot of the reaction mixture was transferred via a gas-tight syringe to a septum-sealed cuvette which had been purged with argon, and the mixture was equilibrated at 37 °C in a water-jacketed cell. The UV-vis spectrum was recorded on a Cary 3 UV-vis spectrophotometer attached to an IBM PSII Model 70 386 computer. To initiate the reaction, a degassed solution of dGTP was added to the cuvette via a gas-tight syringe to a final concentration of 5 mM. Spectra were then recorded every 15 min for 75 min. A control reaction identical to that above but lacking enzyme was also run.

Binding of Cob(II)alamin to C408S RTPR. The reaction mixture contained in a final volume of 500 μ L 200 mM sodium dimethyl glutarate (pH 7.3), 1.6 mM dGTP, 0.2 mM 5'-dA, 280 μ M hydroxocobalamin, and 60 μ M wt or C408S RTPR. The reaction mixture was placed in a 10-mL round-bottom flask equipped with a magnetic stirrer. The flask was sealed with a septum, placed in an ice bath, and degassed by a 20-min purge with argon while being stirred gently. DTT was added to a final concentration of 30 mM via a gas-tight syringe, and the flask was incubated in a 37 °C water bath for 10 min. This procedure allows aquocobalamin to be reduced to cob-(II)alamin. A 400- μ L aliquot was removed from the flask via a gas-tight syringe and applied to a Sephadex G-50 column (1.5 \times 18 cm) which had been equilibrated in 0.1 M argon-purged sodium phosphate buffer (pH 7.3). The column was washed with the argon-purged buffer, and 600- μ L fractions were collected. The spectra of each fraction were recorded on a Cary 3 spectrophotometer (Varian). Control reactions were run in the absence of dGTP and 5'-dA.

Circular Dichroism Spectra. Circular dichroism (CD) spectra were recorded on an AVIV Model 62DS circular dichroism spectrometer (Lakewood, NJ) attached to a CompuAdd 320 computer. Samples included 9 μ M wt RTPR or 12 μ M mutant C408S in 10 mM potassium phosphate buffer (pH 7.2)/1 mM DTT. A baseline containing all

components except protein was run, and then the protein spectrum was obtained from 200 to 260 nm at 37 °C using a 0.5-mm path-length cell.

Analysis of dGTP Binding to C408S RTPR. To remove the DTT and to change buffers, wt or C408S RTPR (0.03 μ mol) was loaded onto a G-50 column (1.5 \times 12 cm) equilibrated in 100 mM sodium phosphate (pH 7.3) at 4 °C and eluted in the same buffer. The protein-containing fractions ($A_{280\text{nm}}$) were collected and pooled. A prehydrated Spectra/por membrane (12 000–14 000 molecular weight cutoff) was allowed to soak in 100 mM sodium phosphate (pH 7.3), 1 mM EDTA, and 0.03% sodium azide for at least 1 h, after which time it was placed as the partition between the halves of an equilibrium dialysis device (Scienceware). The enzyme solution (120 μ L, 15–20 μ M) was pipetted into each well on one side of the equilibrium dialysis device. A 120- μ L aliquot of $[2',3'\text{-}^3\text{H}]\text{dGTP}$ (5–45 μ M, specific activity 4.2×10^6 cpm/ μ mol) in 100 mM sodium phosphate (pH 7.3) was pipetted into each of the wells opposite the protein-containing wells in the equilibrium dialysis device. The equilibrium device was affixed to a vortexer and agitated for 30–40 h at 4 °C. Controls lacking enzyme were run to ensure that equilibration was complete. A 30- μ L aliquot was removed from each well on each side of the membrane, added to 500 μ L of H_2O , and then analyzed by scintillation counting.

RESULTS

Preparation of Site-Directed Mutants. Five cysteines have been targeted for mutagenesis so that a comparison can be made between their phenotypes and those of the corresponding *E. coli* mutants. Cysteines 119, 408, 419, 731, and 736 were converted to serines using the PCR reaction. Additionally, cysteine 305 was changed to a serine using the Amersham *in vitro* mutagenesis kit. All mutant plasmids were transformed into *E. coli* JM101, and individual colonies were screened for the expression of RTPR by SDS-PAGE of saturated overnight cultures (Laemmli, 1970). The integrity of each mutant was confirmed by DNA sequencing. Mutant C736S was observed to contain an additional mutation in which nucleotide 1619 was converted from an A to a T. This change resulted in the conversion of amino acid 540 from a glutamine to a leucine. All other mutants had the expected sequences. The mutant proteins were purified as described previously (Booker & Stubbe, 1993) and portrayed the same characteristics as the wt protein throughout each step of the purification. Each mutant protein, except for C305S, was purified to $\geq 95\%$ homogeneity, with typical yields ranging from 50 to 80 mg per 500 mL of a saturated overnight culture.

At the outset of determining the phenotypes of each of these mutants, it was noticed that there was variability in the level of product production among different preparations of the same mutant protein when the assays were performed with $[^{14}\text{C}]\text{CTP}$ of high specific activity. Control experiments revealed that the variability was the result of small amounts of wt RTPR contamination in the mutant preparation. This contamination resulted from isolating mutant proteins on resin that was previously used to isolate wt RTPR. Although the resin was routinely cleaned with high concentrations of various salts (1 M potassium phosphate or NaCl), these conditions were not stringent enough to remove the trace amounts of wt protein. These results highlight the degree of prudence that must be exercised when attempting to set very low limits of detection for mutants that are expected to have no catalytic activity. The method of cleaning the resin was therefore changed to mild acid and/or base washes, and as a precaution,

each distinct mutant was isolated on resin dedicated to that particular protein.

RTPR Assays. One of the many unique aspects of both the *L. leichmannii* and *E. coli* reductases is the pattern of allosteric regulation that governs which of the four nucleotide substrates is reduced (Reichard, 1988). The *L. leichmannii* enzyme contains a single substrate site which binds each of the four ribonucleoside triphosphates and a separate allosteric site which binds TTP, dGTP, dATP, and dCTP. Appropriate effector binding enhances the rate of substrate turnover ~5-fold (Vitols et al., 1967b). The binding of dGTP stimulates the reduction of ATP. Likewise, dATP stimulates dCTP production, TTP stimulates dGTP production, and dCTP stimulates dUTP production (Beck, 1967; Chen et al., 1974). This elaborate array of allosteric control of substrate turnover is abrogated with the addition of high concentrations of certain ions such as acetate (Jacobsen & Huennekens, 1969). One molar NaOAc allows each of the four NTPs to be reduced to dNTPs in the absence of any effectors with turnover numbers that are almost identical to those observed in the presence of effectors.

In addition to the requirement for an allosteric effector, RTPR also requires a reductant that rereduces the active site disulfide generated concomitant with substrate reduction. It is generally believed that, *in vivo*, TR provides RTPR with reducing equivalents which come ultimately from NADPH in a process that is mediated by the flavoprotein TRR (Moore et al., 1964). *In vitro*, however, the *E. coli* TR (which has been cloned and overexpressed) can serve this purpose. Alternatively, reducing equivalents can be provided by small dithiol molecules such as dihydrolipoic acid and dithiothreitol (Beck et al., 1966).

Because of the complexity of the reaction with regard to the mechanism of substrate reduction and allosteric activation, we have chosen to investigate ribonucleotide reduction under a defined set of conditions. The reduction of CTP has been investigated in the presence of dATP or sodium acetate, using the *E. coli* TR or DTT as the reductant. Each mutant (or wt protein) is thus assayed under four different sets of conditions. The choice of [¹⁴C]CTP as substrate was dictated by the ease of product analysis, and this substrate was synthesized from the corresponding [¹⁴C]CDP using pyruvate kinase in conjunction with phosphoenolpyruvate as the phosphate donor (Worthington, 1988).

Characterization of C731S, C736S, and the Double Mutant C731 and 736S. Previous studies of Mao et al. (1989, 1992a) using C754S and C759S R1 mutants of the *E. coli* RDPR suggested that the function of these C-terminal cysteines was to shuttle reducing equivalents from TR into the active site via disulfide interchange. The similarity in sequence between cysteines 754 and 759 of the *E. coli* RDPR and the C-terminal cysteines 731 and 736 of RTPR, in conjunction with biochemical studies of Lin et al. (1987), suggested that these cysteines in the *Lactobacillus* enzyme might serve a similar function. If indeed these cysteines provide a pathway for TR to rereduce the active site disulfide of RTPR, then two phenotypic characteristics would be predicted. In the presence of the TR/TRR/NADPH reducing system, a single turnover of CTP to dCTP would be expected. Subsequent turnovers would be prohibited since the vehicle for shuttling reducing equivalents into the active site has been destroyed. On the other hand, small organic dithiols such as DTT or dihydrolipoic acid might be expected to bypass these C-terminal cysteines, providing reducing equivalents directly to the active site disulfide.

Table 1: Phenotypes of the C-Terminal RTPR Mutants

| protein (condition) | specific activity | | single turnover (equiv of dCTP) |
|---------------------------|---|---|------------------------------------|
| | DTT ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) | TR/TRR/NADPH ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) | |
| wild type (acetate) | 1.5 ^a | 1.2 | 1.4 |
| wild type (dATP) | 0.1 | 1.0 | 1.5 |
| C731S (acetate) | 0.9 | $<5 \times 10^{-5}$ | 0.8 |
| C731S (dATP) | 0.095 | $<5 \times 10^{-5}$ | 0.8 |
| C736S (acetate) | 1.6 | $<5 \times 10^{-5}$ | 0.6 |
| C736S (dATP) | 0.13 | $<5 \times 10^{-5}$ | 0.6 |
| C731 and 736 (acetate) | 1.8 | $<5 \times 10^{-5}$ | 0.7 |
| C731 and 736S (dATP) | 0.09 | $<5 \times 10^{-5}$ | 0.7 |

^a Freezing and thawing of RTPR results in loss of activity from 1.5 to 1.0 unit/mg.

Our model predicts that for wt RTPR under single-turnover conditions a maximum of 2 equiv of dCTP should be produced if the C-terminal cysteines can completely rereduce the active site disulfide subsequent to production of the first dCTP. In the same fashion, each of the C-terminal mutants should only be capable of producing 1 dCTP. This prediction was tested by prereducing the enzyme with DTT and then removing the reductant by size-exclusion chromatography. The enzyme was then treated with the substrate in the absence of either reductant and in the presence of dATP or NaOAc. These experiments indicate that C731S, C736S, and C731 and 736S RTPRs all result in the production of 0.6–0.8 equiv of dCTP (Table 1). The wt RTPR, on the other hand, results in the production of 1.4 equiv of dCTP. The substoichiometric amount of dCTP for each of the mutants and the wt RTPR relative to the predicted value is not understood. From control studies we believe that this is not a consequence of partial oxidation of the prereduced enzyme before the initiation of the one-turnover experiment. It may be significant that in analogous experiments using the *E. coli* RDPR 2.6 dCDPs are observed per R1 dimer in which four sets of cysteines are available to produce a maximum of 4 dCDPs. For each of the *E. coli* C-terminal mutants, as well as the C-terminal double mutant, equivalents of dCDP ranged from 0.9 to 1.2 in single-turnover experiments. Despite the fact that the number of equivalents of dNTP per equivalent of enzyme is lower than expected, it is significant that, in each C-terminal mutant, the number is approximately one-half of that observed with the wt RTPR.

Experiments carried out in the presence of reductant corroborate our hypothesis. Wt RTPR has a specific activity of 1–1.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ when assayed in the presence of the thioredoxin reducing system using the allosteric effector (dATP) for CTP reduction or in the presence of NaOAc. Subsequent to the initial turnover, each of the C-terminal C → S mutants produces dCTP at a rate that is less than 5×10^{-5} that of the wt RTPR. This rate represents the lower limit of detection of this particular assay. When the assay is conducted using DTT as the reductant, the rate of turnover for each of the mutants under a defined set of conditions is similar to that of the wt protein. These results provide strong support for the integrity of the enzyme (since it can generate dCTP) and the role of the C-terminal thiols as redox shuttles.

Studies of the kinetics of the single-turnover reaction using HPLC analysis to examine product production provided some unexpected results. The amount of dCTP in the first time point (1 min) is equivalent to the amount observed in the 20-min time point. However, a small amount of cytosine and

Table 2: Equivalents of dC, cytosine (Cyt), and the Mystery Peak (Z) as a Function of Time

| | 1 min | | | 20 min | | |
|-----------------|-----------------------------|-----------------|---------------|----------------|-----------------|---------------|
| | equiv ^a of dC | equiv of Cyt | equiv of Z | equiv of dC | equiv of Cyt | equiv of Z |
| protein (NaOAc) | | | | | | |
| wt | 1.5 | 0.03 | <0.01 | 1.4 | 0.2 | 0.2 |
| C731S | 0.8 | 0.02 | <0.04 | 0.8 | 0.1 | 0.3 |
| C736S | 0.6 | 0.03 | <0.03 | 0.6 | 0.2 | 0.3 |
| C731 and 736S | 0.7 | 0.02 | <0.02 | 0.7 | 0.3 | 0.2 |
| protein (dATP) | | | | | | |
| wt | 1.5 | 0.05 | <0.01 | 1.6 | 0.4 | <0.06 |
| C731S | 0.8 | 0.04 | <0.04 | 0.8 | 0.2 | <0.2 |
| C736S | 0.6 | 0.03 | <0.03 | 0.6 | 0.2 | <0.1 |
| C731 and 736S | 0.7 | 0.1 | <0.04 | 0.7 | 0.3 | <0.04 |

^a equiv = equivalents per mole of RTPR.

some yet uncharacterized species which elutes at 14–15 min (methods section) is produced in a slow time-dependent fashion (Table 2). This uncharacterized species is much more prevalent in assays conducted in the presence of NaOAc and is approximately equal to the amount of cytosine observed after a 20-min incubation. The amount of cytosine and this uncharacterized peak is usually negligible in the first time point of the assay, suggesting that it might be due to chemistry subsequent to active site disulfide formation. In order to test this hypothesis, wt RTPR was treated with substrate for 2 min at 37 °C in a single-turnover experiment (absence of reductant), and then the protein was separated from the small molecules by size-exclusion chromatography. The preoxidized enzyme was then incubated with the radioactive substrate and analyzed for the time-dependent production of dCTP, cytosine, and the unknown species. After 40 min, ~0.29 equiv of cytosine and 0.28 equiv of the unknown species were produced, and there was no detectable dC. The kinetics of cytosine and the unknown species are essentially linear for the first 20 min and then taper off during the subsequent 20 min. The structure of this unknown species is presently under investigation. A slow formation of cytosine using oxidized *E. coli* RDPR has also been observed (G. Yu, unpublished results). These results are also very similar to previous studies of the interaction with CIUDP with oxidized *E. coli* RDPR. The oxidized enzyme also catalyzes formation of cytosine from CIUDP at one-tenth the rate of the reduced RDPR (Ator & Stubbe, 1985).

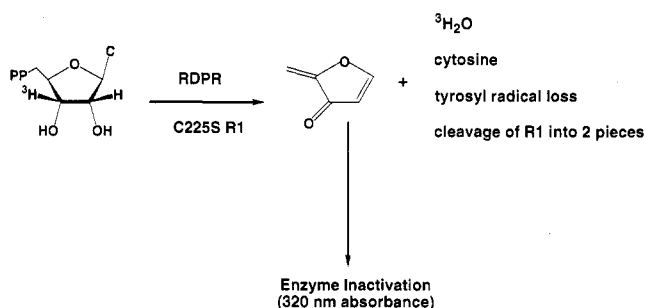
Assays with C119S and C419S RTPR. Cysteines 119 and 419 are proposed to be the cysteines in the active site of RTPR that are directly involved in substrate reduction. If indeed this is the case, our model predicts that, upon mutating these amino acids to serines and incubating the mutant enzymes with CTP, absolutely no product (dCTP) should be produced. Detailed studies with the mechanism-based inhibitor CIUTP, as well as with the C225S R1 and C462S R1 active site mutants of the *E. coli* RDPR, suggest that, in the event that 3' C–H bond cleavage is uncoupled from the transfer of reducing equivalents to the substrate or substrate analog, the substrate or analog becomes a mechanism-based inhibitor of the enzyme (Harris et al., 1984; Ashley et al., 1988; Mao et al., 1989, 1992a–c). A set of experiments under various assay conditions was therefore carried out to determine if cytosine or dCTP is produced when these mutants are incubated with RTPR. The results of these experiments are summarized in Table 3. As predicted for cysteines providing reducing equivalents required for substrate reduction, no dCTP is produced that is above our experimental limit of detection (0.02–0.08 dCTP per equivalent of RTPR).

Table 3: Phenotypes of the Active Site C → S Mutants: Equivalents of Product per Equivalent of RTPR

| mutant and assay condition | equiv of cytosine | equiv of deoxycytidine |
|----------------------------|-------------------|------------------------|
| C119S (30 min at 37 °C) | | |
| DTT and NaOAc | 14 | <0.02 |
| DTT and dATP | 62 | <0.02 |
| TR/TRR and dATP | 15 | <0.02 |
| TR/TRR and NaOAc | 0.6 | <0.08 |
| C419S (60 min at 37 °C) | | |
| DTT and NaOAc | 10 | <0.02 |
| DTT and dATP | 55 | <0.02 |
| TR/TRR and dATP | 19.7 | <0.02 |
| TR/TRR and NaOAc | 2.5 | <0.02 |

Furthermore, on the basis of our previous studies with CIUTP (eq 1), removal of the active site reducing equivalents should result in conversion of CTP to 3'-keto-2'-deoxycytidine 5'-triphosphate, which would subsequently collapse nonenzymatically to generate cytosine, PPP_i, and a 2-methylene-3-(2H)-furanone which could alkylate the enzyme. The alkylation has been shown to be accompanied by a $\Delta A_{320\text{nm}}$ on the protein. As indicated in Table 3, cytosine release is observed under all assay conditions. Furthermore, isolation of the protein from the assay mixtures containing the thioredoxin reducing system revealed an absorption feature at 320 nm (data not shown). From the numbers in Table 3, it can be seen that the amount of cytosine released with DTT as the reductant is severalfold greater than that observed with the TR reducing system under the same set of conditions. Again, on the basis of our previous studies with CIUTP, this difference may be attributed to the ability of DTT to trap the highly reactive 2-methylene-3-(2H)-furanone before it alkylates the enzyme.

Lastly, when either C419S or C119S is treated with CTP in the presence of DTT under anaerobic conditions, AdoCbl is partially destroyed, resulting in the production of cob(II)-alamin (B_{12r}), which can be observed spectrophotometrically at 477 nm (data not shown). The details of the cofactor destruction are currently under investigation; however, it is analogous to loss of the tyrosyl radical with the corresponding *E. coli* active site mutants (Mao et al., 1989, 1992b) (eq 2).



The ability to catalyze cofactor destruction in conjunction with the observation of cytosine release suggests that each of these mutants is structurally intact, as they are able to bind AdoCbl and CTP and catalyze chemistry ascribed to abstraction of the 3' hydrogen atom of the substrate. The inability of these mutants to catalyze dCTP production strongly supports their function as the direct providers of reducing equivalents during substrate reduction.

Characterization of C408S. Cysteine 408 of RTPR was targeted for mutagenesis on the basis of sequence homology with C439 of the *E. coli* RDPR (Booker & Stubbe, 1993).

Previous mutagenesis studies on the *E. coli* enzyme have suggested that C439 might be the amino acid residue responsible for initiating the reduction process by abstracting the 3' hydrogen atom of the substrate (Mao et al., 1992c). If C408 of RTPR is the counterpart of C439 of RDPR, then our model predicts that absolutely no dCTP or cytosine should be produced, since both require 3' C-H bond cleavage for their production. The reaction of C408S RTPR with CTP was therefore investigated using both reductants in combination with either the allosteric effector or NaOAc. Under all four sets of conditions, neither dCTP nor significant amounts of cytosine were produced in a 30-min incubation at 37 °C. Given the large amounts of enzyme (24 μ M) and the use of CTP of high specific activity, this mutant makes product at a rate that is less than 2×10^{-5} times that of the wt protein. This is our lower limit of detection in this assay. Thus, C408S RTPR is inactive with respect to nucleotide reduction.

The coenzyme B₁₂-requiring reductases are unique in that they catalyze two additional reactions which can be used as well to investigate the importance of C408. The first reaction is the equilibration with water of the 5'-methylene hydrogens of AdoCbl. If the cofactor is synthesized with tritium at the 5' position, the enzyme catalyzes the release of all of the tritium to water (Abeles & Beck, 1967; Hogenkamp et al., 1968). Both wt RTPR and mutant C408S were assayed for their ability to catalyze this exchange. Wt RTPR (0.06 nmol) catalyzes release of ~ 5500 cpm of $^3\text{H}_2\text{O}$ in a 10-min incubation at 37 °C. However, 6 nmol of mutant C408S RTPR releases 60 cpm of $^3\text{H}_2\text{O}$ in a 30-min incubation at 37 °C, which is virtually indistinguishable from the results with the control in the absence of enzyme. The results indicate that C408S RTPR has an activity for the exchange reaction that is $<0.006\%$ that of wt RTPR. The second reaction catalyzed by RTPR is a slow decomposition of the cofactor to give 5'-dA and cob(II)alamin (B_{12r}) when the experiment is conducted under anaerobic conditions (Yamada et al., 1971). Under the set of conditions described in the Materials and Methods section, 0.56 equiv of B_{12r} is formed with wt RTPR after a 75-min incubation at 37 °C, whereas no B_{12r} is detectable with mutant C408S. The limit of detection for this assay is 0.04 equiv of B_{12r}. Thus, C408S RTPR is unable to catalyze two additional reactions characteristic of wt RTPR.

Given that C408S RTPR possesses no detectable catalytic activities, several experiments were carried out in an effort to show that the protein is properly folded. Circular dichroism spectra of both wt RTPR and C408S RTPR were recorded, and both proteins exhibited almost identical ellipticities when normalized for the amount of enzyme used in each determination (data not shown).

A more definitive experiment to address the folding of C408S RTPR is to examine its ability to bind AdoCbl, allosteric effector (dGTP), and substrate. The ability of wt RTPR to catalyze a slow breakdown of AdoCbl in the presence of dNTP and DTT has previously precluded an accurate determination of a binding constant for the cofactor. The observation that C408S RTPR does not catalyze this decomposition made it a prime candidate to allow a measurement for the first time of a K_d for AdoCbl. Efforts to make this determination using filter binding assays (Ormö & Sjöberg, 1990) were, however, unsuccessful. The data were very scattered and the K_d was high.

Previous studies of Yamada et al. (1971) had indicated that wt RTPR can bind the putative intermediates in the RTPR-catalyzed reaction [cob(II)alamin and 5'-dA] in the presence of dGTP and DTT. These results suggested that the

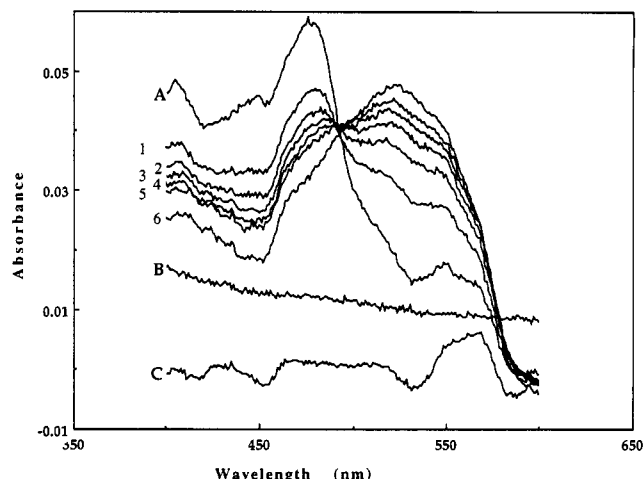


FIGURE 1: Analysis of cob(II)alamin binding to C408S RTPR. (A) Cob(II)alamin binding in the presence of dGTP and 5'-dA. Additional spectra were obtained 2 min (1), 4 min (2), 6 min (3), 8 min (4), 10 min (5), and 12 min (6) after the initial scan. Cob(II)alamin binding (B) in the absence of dGTP and (C) in the absence of 5'-dA.

apparent low K_m s for AdoCbl may be a reflection of tight binding of the "intermediates" and not the intact cofactor. C408S RTPR's inability to catalyze formation of cob(II)-alamin is perhaps due to the required coupling of this carbon-cobalt bond homolysis to formation of the protein radical (X \cdot), where X is proposed to be C408. This uncoupling would then prevent detection of tight binding of AdoCbl; but if the model is correct, then binding of cob(II)alamin and 5'-dA should be detected. To test this hypothesis, C408S RTPR was therefore incubated with cob(II)alamin, 5'-dA, and dGTP and then passed through a Sephadex G50 column, and the UV-vis spectrum of the protein was recorded (Figure 1). The results show that ~ 0.5 equiv is bound to the mutant protein, and control experiments reveal that both 5'-dA and dGTP are required for this binding (Figure 1). A similar experiment with wt RTPR revealed ~ 0.25 equiv of cob(II)alamin bound. The quantitation and structural characterization of the cob(II)alamin were carried out by allowing it to oxidize to aquocobalamin (Figure 1). These studies establish the ability of C408S RTPR to bind cofactor in its "active" form as well as the effector dGTP. Binding of the latter was confirmed using equilibrium dialysis with [$1',2',3\text{-}^3\text{H}$]dGTP. A Scatchard plot revealed a K_d of $4 \pm 1.3 \mu\text{M}$ and 0.88 ± 0.3 binding site. These results compare with values of $5.8 \pm 0.4 \mu\text{M}$ and 0.79 ± 0.06 for wt RTPR determined by the same procedure and $1.73 \pm 0.07 \mu\text{M}$ and 1.07 ± 0.02 previously determined for RTPR isolated from *L. leichmannii* (Singh et al., 1977). It is unclear why less than one site is observed for the wt RTPR, although Chen et al. (1974) observed 0.85 site for dGTP using wt enzyme at 25 °C. It is interesting to note that this number is similar to the number of active sites determined by the one-turnover experiments with the C-terminal mutants of RTPR. Thus, while C408S RTPR possesses no detectable catalytic activity, it is still capable of binding effectors and putative intermediates resulting from AdoCbl.

Characterization of C305S RTPR. As a control experiment to ensure that our choice of mutants was not fortuitous, C305 was converted to a serine. This mutant was partially purified and was shown to have a specific activity $\sim 60\%$ that of wt RTPR. Furthermore, characterization of the products produced on interaction with CTP revealed only dCTP.

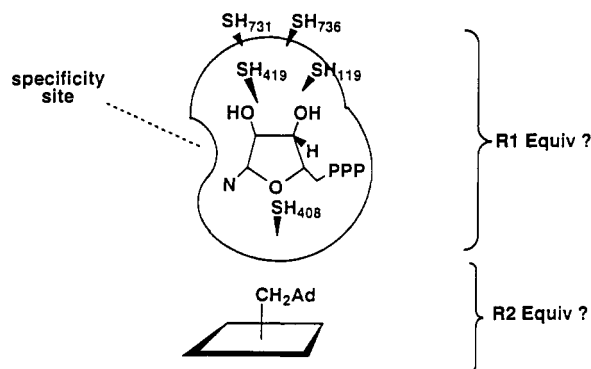


FIGURE 2: Postulated model for the role of five cysteines in nucleotide reduction.² Sequence homology searches with other B₁₂-requiring enzymes suggest that the AdoCbl binding site lies between amino acids 169 and 413 (Marsh & Holloway, 1992).

DISCUSSION

The recent cloning and expression of RTPR (Booker & Stubbe, 1993) has allowed us to investigate the complex role of the cysteines involved in nucleotide reduction using site-directed mutagenesis. The studies presented in this paper, in conjunction with earlier studies on the *E. coli* RDPR, provide strong support for a model in which five cysteines are required for nucleotide reduction in each of these enzymes (Figure 2).² The active site thiols of RTPR and their *E. coli* counterparts are proposed to be C408 (C439), C119 (C225), and C419 (C462). Cysteines 119 and 419 are the residues which become oxidized concomitant with substrate reduction. Cysteine 408 is postulated to be oxidized to a thiyl radical by the products resulting from the homolysis of the carbon-cobalt bond of AdoCbl. It is this thiyl radical which is then proposed to initiate substrate reduction by abstracting its 3' hydrogen atom. Two additional cysteines, C731 and C736 (C754 and C759 in the *E. coli* RDPR), are proposed to shuttle reducing equivalents into the active site disulfide via disulfide interchange from the *in vivo* protein reductant TR.

Both the *E. coli* and *L. leichmannii* reductases have C-terminal tails with similar sequence contexts. The studies

RTPR: DLELVDQTD_C731EGGAC736PIK

RDPR: DLVPSIQDDGC754ESGAC759KI

with the C → S mutants of these residues, besides providing strong support for their function, also shed light on the protein's dynamics. Comparison of the results using TR and DTT as reductants suggests that this C-terminal tail is flexible and that the flexibility is altered by the presence of allosteric effectors. For example, DTT can provide reducing equivalents directly to the active site cysteines, by-passing the C-terminal tail which must be sufficiently dynamic to allow access to the active site. Second, dATP, the allosteric effector required for CTP reduction, modulates the flexibility of the C-terminal tail as evidenced by a drop in the reduction rate to one-tenth of that observed in the presence of NaOAc. Similar results have previously been reported with the *E. coli* RDPR and these same reductants, again indicating a dynamic C-terminal tail in both of these systems (Mao et al., 1992a).

As indicated earlier, the *L. leichmannii* and *E. coli* enzymes display very little sequence homology with respect to each

other (Booker & Stubbe, 1993). This hampered the process of deciding which of the ten cysteines in RTPR might be directly involved in the reduction of the NTP substrate. An important clue to their assignment came from our earlier biochemical studies which suggested that C119 was able to undergo disulfide interchange with the C-terminal cysteines (Lin et al., 1987). The basis for selecting C419, however, was not as obvious and requires additional comment. C419 was targeted on the basis of its primary sequence relationship relative to C408, 11 amino acids displaced toward the C-terminal end. The corresponding cysteine in the *E. coli* RDPR, C462, is 23 amino acids displaced from C439 in the same sense. A comparison of the dinuclear-iron center reductases from 11 sources suggests that even though C462 is conserved, its spacing relative to C439 is variable (Eriksson & Sjöberg, 1989; Chakrabarti et al., 1993). In human, yeast, and vaccinia virus RDPRs, these cysteines are separated by 15 residues, while in the Epstein Barr virus, they are displaced by 14 residues. The C⁴¹⁹NL sequence found in RTPR has also been identified in 9 of 11 iron-dependent reductases, with the *E. coli* and phage T4 reductases having the sequence CTL. Thus, C419 and C119 were targeted for mutation, the former being considered as the active site equivalent to *E. coli* C462. As a control experiment, an additional cysteine, 305, was also mutated to a serine.

The phenotypes of the serine mutants created from the two cysteines (119, 419) proposed to directly provide reducing equivalents to the nucleotide substrate are very similar to those previously reported for the corresponding *E. coli* RDPR equivalents (225 and 462). In contrast to these previous studies, however, the present studies are unambiguous, as our heterologous expression system has avoided problems associated with contaminating wt protein. The absence of dCTP and the presence of cytosine when these mutants are incubated with CTP indicate, as our mechanism has proposed, an uncoupling of the reduction step from the initiation step involving hydrogen atom abstraction from the 3' position of the nucleotide. The altered chemistry, including cytosine release, which occurs when these steps are uncoupled has been well documented (Mao et al., 1992b). The results in Table 3 and the control experiment with C305S RTPR suggest that C419 and C119 are in fact in the active site of RTPR.

The model in Figure 2 also predicts that C408 should be close within three-dimensional space to C419 and C119. Its importance has been identified by sequence context and by results with the *E. coli* C439S mutant (Mao et al., 1992c).

RTPR: TNPC⁴⁰⁸GEISLA

RDPR: SNLC⁴³⁹LEIALP

As outlined above, this RTPR mutant is inactive with respect to catalysis of nucleotide reduction, washout of ³H from [5'-³H]AdoCbl, and breakdown of AdoCbl to cob(II)alamin and 5'-dA. To conclude from these studies that C408 is essential for catalysis, one needs to demonstrate that the mutant protein is properly folded. Measurement of substrate, effector, or cofactor binding is one way to address this problem in the absence of a three-dimensional structure. With C408S RTPR, however, no binding constant for AdoCbl could be measured under a variety of conditions varying the reductants, effectors, and substrates in all combinations and permutations. While this result was initially perplexing, it perhaps should have been anticipated on the basis of previous studies of Yamada et al. (1971) that revealed that cob(II)alamin and 5'-dA bind

² The authors thank Professor Rowena Matthews for helpful discussions concerning the location of AdoCbl binding to RTPR based on the methionine synthase structure and Robert Suto for his alignment of RTPR with methionine synthase.

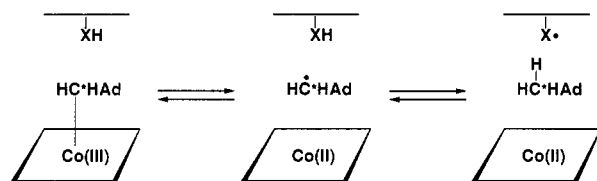


FIGURE 3: Model for the generation of a protein radical upon binding AdoCbl. XH is proposed to be C408 of RTPR.

tightly to RTPR and competitively with respect to AdoCbl in the presence of an allosteric effector and reductant. This coupling of carbon–cobalt bond homolysis of AdoCbl to formation of the protein radical (Figure 3) with the wt protein could explain the observed K_m of 0.3 μ M for AdoCbl (Blakley, 1978) even though the K_d for AdoCbl, under similar conditions, is too large to measure. C408S RTPR cannot catalyze carbon–cobalt bond homolysis. However, if the active site is intact in this mutant, it should be able to bind 5'-dA and cob(II)-alamin. While K_d s have not yet been measured for either of these compounds due to the unavailability of labeled materials, the studies using Sephadex G-50 chromatography (Figure 1) indicate that the mutant can bind the "active" form of the cofactor if and only if 5'-dA and effector are present. These binding studies, the ability to bind effector, dGTP, and the inability of C408S RTPR to catalyze any reactions suggest that C408 plays an essential role in catalysis and is consistent with its role as the X• in our proposed mechanism.

These studies support our hypothesis that AdoCbl required by RTPR for catalysis is equivalent to the R2 subunit of the *E. coli* RDPR. Studies of Tamao and Blakley (1973) have established that, subsequent to AdoCbl binding, RTPR catalyzes homolysis of its carbon–cobalt bond, resulting in the production of cob(II)alamin and a putative 5'-deoxyadenosyl radical (5'-dA•). This state of RTPR would be equivalent to the tyrosyl radical and dinuclear-iron center of R2 *E. coli* RDPR. In neither case, however, is the initially generated radical responsible for hydrogen atom abstraction from the substrate. In the case of RDPR, the X-ray structure and biophysical studies suggest that nucleotide reduction is initiated by long-range electron transfer from R1 to R2 to generate a thiyl radical of C439 (Stubbe, 1990a; Mao et al., 1992a; Nordlund & Eklund, 1993). Our studies on RTPR required, as with the *E. coli* RDPR, that the putative 5'-dA• does not directly mediate hydrogen atom abstraction from the substrate (Ashley et al., 1986). The mutagenesis studies reported herein suggest that the putative 5'-dA• generates a protein radical, C408, which then initiates catalysis.

What is striking about the studies reported in this paper is the remarkable similarities of the phenotypes of the five mutants of RTPR in comparison with those of RDPR. In spite of the fact that the quaternary structures, primary structures, and cofactor requirements for these reductases are unique, they appear to have evolved strikingly similar chemical mechanisms as well as a similar role for the five cysteines in catalysis. These data provide strong support for our original hypothesis that the cofactors act as radical chain initiators, generating a protein radical sufficiently removed from the cofactor binding site such that the redox-active cysteines required for the reduction process would not interfere with the radical-initiator cofactor. Efforts are now focused on obtaining evidence that unambiguously defines the protein radical, X•, in our proposed mechanism and the mechanism by which it is generated.

SUPPLEMENTARY MATERIAL AVAILABLE

A description of the preparation of all RTPR mutants and a table listing primers used for mutagenesis (4 pages). Ordering information is given on any current masthead page.

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