A Model for the Role of Multiple Cysteine Residues Involved in Ribonucleotide Reduction: Amazing and Still Confusing

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ABSTRACT: Ribonucleotide reductase from *Escherichia coli* catalyzes the conversion of nucleotides to deoxynucleotides. Multiple cysteines have been postulated to play a key role in this process. To test the role of various cysteines in nucleotide reduction, a variety of single and double mutants of the R1 subunit were prepared: C754S, C759S, C754–759S, C462S, C462A, C230S, and C292S. Due to the expression system, each mutant contains small amounts of contaminating wt-R1 (estimated to be 1.5–3% based on activity). An epitope tagging method in conjunction with anion exchange chromatography was used to partially resolve the mutant R1 from the wt-R1. The interaction of these mutants with the normal substrate was studied, which allowed a model to be proposed in which five cysteines of the R1 subunit of RDPR play a role in catalysis. C754S and C759S R1s catalyze CDP formation at rates similar to wt-R1 when DTT is used as a reductant. However, when thioredoxin (TR)/thioredoxin reductase (TRR)/NADPH is used as reductant, the rates of dNDP production are similar to those expected for contaminating wt-R1 present as a heterodimer with the mutant. The impaired nature of these mutants with respect to reduction by TR suggests that their function is to transfer reducing equivalents from TR to the active site disulfide of R1 produced during NDP reduction. Single-turnover experiments, designed to avoid the problem of contaminating wt-R1, also support this role for C754 and C759. The double serine mutant of 754 and 759 has catalytic activity with DTT that is one-third the rate of wt-R1 with thioredoxin. C225 and C462 are thought to be the active site cysteines oxidized concomitantly with NDP reduction. Conversion of these cysteines to serines results in R1 mutants which convert the normal substrate into a mechanism-based inhibitor. C462SR1 upon incubation with R2 and [3′-^{3}H,U-^{14}C]UDP results in uracil release, ^3H_2O production, ^{3}H,^{14}C-labeled protein which has an absorbance change at 320 nm, and slow loss of the tyrosyl radical on R2. The isotope effect (k_H/k^3_H) on 3′ carbon–hydrogen bond cleavage is 1.7. This sequence of events is independent of the reductant, consistent with the postulate that C462 is an active site thiol. The C462AR1 has properties similar to C462SR1. Several additional mutant R1s, C230SR1, and C292SR1 were shown to have activities similar to wt-R1 with both TR/TRR/NADPH and DTT.

INTRODUCTION

Ribonucleotide reductases play a central role in DNA biosynthesis, catalyzing the conversion of nucleotides to deoxynucleotides (Thelander & Reichard, 1979; Stubbe, 1990; Eriksson & Sjöberg, 1989). The *Escherichia coli* ribonucleoside diphosphate reductase (RDPR) is composed of two subunits. R1 is a homodimer of M_r = 171.4 and R2 is a homodimer of M_r = 86.5. The R1 subunit contains binding sites for the nucleoside 5′-diphosphate (NDP) substrates and the nucleoside and deoxynucleoside triphosphate allosteric effectors, in addition to the cysteines that become oxidized concomitantly with substrate reduction. On the basis of extensive studies with [3′-^{2}H]- and [3′-^{3}H]NDPs and mechanism-based inhibitors, a mechanism for substrate reduction (Scheme I) has been proposed (Stubbe, 1990). This model predicts that a one-electron-oxidized amino acid residue on the protein (X•) becomes reduced concomitantly with cleavage of the 3′ carbon–hydrogen bond of the substrate. Subsequent to a complex series of transformations involving loss of H_2O followed by numerous electron and proton transfers, a 2′-deoxynucleoside 5′-diphosphate (dNDP) is produced and the amino acid residue XH is reoxidized to X•. Although studies using wild-type enzyme and isotopically labeled NDPs (Stubbe et al., 1983) and 2′-substituted 2′-dNDP derivatives have been extremely useful in confirming certain aspects of the mechanism, they have failed to reveal a direct correlation between 3′-CH bond cleavage of the NDP substrate and reduction of a protein radical (Thelander et al., 1976; Sjöberg et al., 1983; Ator & Stubbe, 1985; Salowe et al., 1987; Ator et al., 1984).

As an alternative to the use of substrate analogs to study this reaction mechanism, we and others have decided to use protein analogs, site-directed mutants, to investigate this problem (Mao et al., 1989; Aberg et al., 1989). Specifically, we postulated that if each redox-active cysteine could be changed into a redox-inactive residue, the flow of electrons from enzyme to substrate might be blocked, which in turn might allow the first step of this complex mechanism to be investigated. We also hoped such studies would provide insight into the function of the additional cysteines that our earlier studies (Mao et al., 1989; Lin et al., 1987) and more recent studies of Aberg et al. (1989) have suggested are required for catalysis.

Two independent methods, both intended to identify the catalytically essential cysteine residues from among the 11 present in each monomer, suggested that multiple cysteine residues might be involved in catalysis. The biochemical studies of Lin et al. (1987), designed to radiolabel only the cysteines which were oxidized concomitantly with substrate reduction, unexpectedly revealed five cysteines, C225, C230, C462, C754, and C759, in peptides containing radiolabels. These results suggested that these cysteines in R1 are spatially close enough to the active site and to each other to undergo disulfide interchange. It was not possible, however, to unambiguously conclude which pair (or pairs) of these cysteines directly reduced the substrate. Sequence alignment

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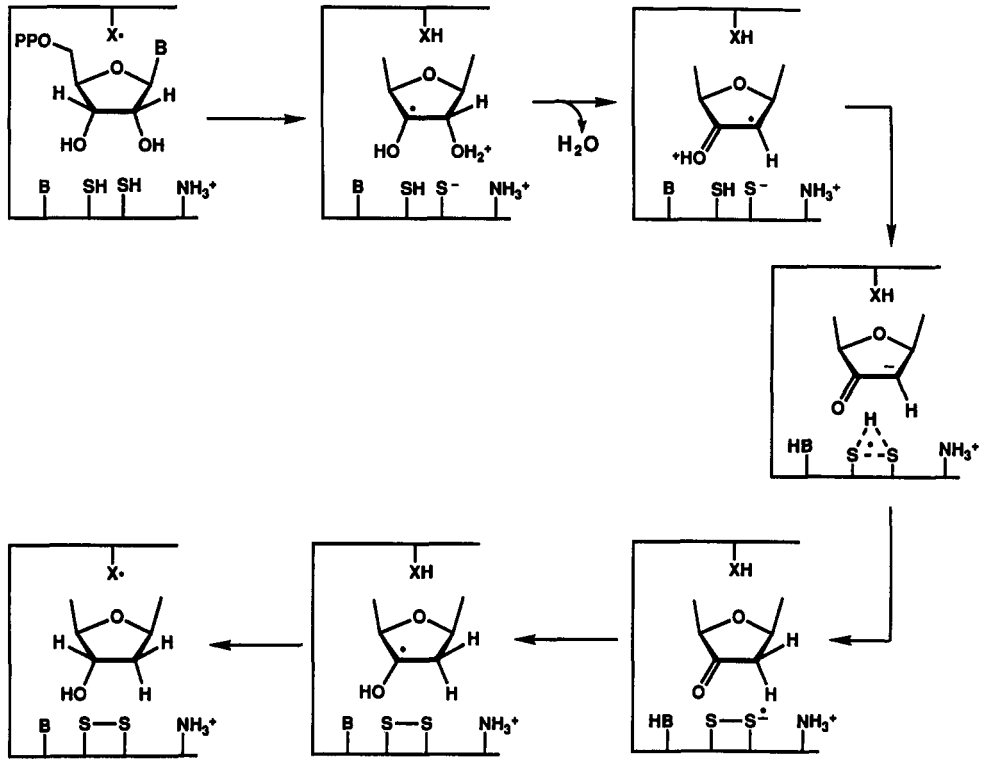
Scheme I



of R1 subunits from eight different sources (Nilsson et al., 1988) also suggested that multiple cysteine residues might be important. Three cysteine residues, C225, C439, and C462, were found to be conserved. Two additional cysteines, C754 and C759 in *E. coli*, were found near the C-terminus of all R1s, although neither their exact position nor their position relative to one another is conserved. Taken together, the biochemical studies and the sequence alignment provide six different candidates for site-directed mutagenesis. The preparation and preliminary characterization of serine mutants of two of these candidates, C225 and C759, have previously been described (Mao et al., 1989). The present paper reports the preparation and characterization of the additional site-specific mutants C230S, C462S, C462A, C754S, and the double mutant C754S and C759S. Additional characterization of C759SR1 is also reported. The accompanying papers describe further findings with the mutant C225SR1 and the characterization of C439SR1. The results reported herein have allowed us to classify these mutants into three general categories based upon their catalytic activity with CDP in the presence of either the natural reducing system thioredoxin/thioredoxin reductase/NADPH or the low molecular weight organic reductant dithiothreitol (DTT). Evidence to support the role in catalysis of each cysteine and a mechanistic model for nucleotide reduction which incorporates the proposed function of each are presented.

MATERIALS AND METHODS

[U-^{14}C]Cytidine 5′-diphosphate (CDP; specific activity 532 mCi/mmol) was purchased from New England Nuclear and diluted with unlabeled CDP (Sigma). [3′-^{3}H,U-^{14}C]UDP was prepared by mixing [3′-^{3}H]UDP, synthesized by standard procedures (Stubbe & Ackles, 1980), with [U-^{14}C]UDP, synthesized from [U-^{14}C]uridine (New England Nuclear; specific activity 521 mCi/mmol). Adenosine 5′-triphosphate (ATP) and thymidine 5′-triphosphate (TTP) were purchased from Sigma. Dithiothreitol (DTT) was purchased from United States Biochemicals; concentrations were verified using Ellman's reagent (Jocelyn, 1987). Monoclonal antibody 12CA5 was obtained from Berkeley Antibody Co. All other reagents and chemicals were of the highest purity available.

UV/visible absortion spectra were recorded on a Hewlett-Packard 8452A spectrophotometer. All scintillation counting was performed on a Packard 1500 liquid scintillation analyzer with Scint-A XF (Packard) as scintillation fluid. HPLC chromatography was performed on an Altex 110A with an Altex 420 controller and a Hitachi 100-40 detector. SDS/PAGE was performed as described by Laemmli (1970).

Strains and Enzymes. Wild-type *E. coli* RDPR subunit R2 (ϵ_{280nm} = 130 500 M^{-1} cm^{-1}) was isolated from the overproducing strain N6405/pSPS2 (Salowe & Stubbe, 1986). Wild-type RDPR subunit R1 was isolated from overproducing strain C600/pMB1, ϵ_{280nm} = 189 000 M^{-1} cm^{-1} (Salowe & Stubbe, 1986). *E. coli* thioredoxin (TR) and thioredoxin reductase (TRR) were isolated from overproducing strains SK3981 [specific activity 36 units/mg; Lunn et al. (1984)] and K91/pMR14 [specific activity 1000 units/mg; Russel and Model (1985)], respectively. Endonucleases, T_4 DNA ligase, and polynucleotide kinase were purchased from New England Biolabs. DNAse-free RNAse was purchased from Boehringer Mannheim; *E. coli* alkaline phosphatase was purchased from Sigma.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by the phosphorothioate method using a kit purchased from Amersham (Taylor et al., 1985). Oligonucleotide primers were synthesized using a Biosearch 8600 (Milligen Biosearch, Millipore) DNA synthesizer and purified either by reverse-phase HPLC or by polyacrylamide gel electrophoresis. Specific primer sequences were as follows: for C230S, 5′-CTGATCGAGTCCGGAGACAGC; for C462S, 5′-ATCGCGCTGTCAACGCTGTCT; for C462A, 5′ATCGCGCTGGCTACGCTGTCT; for C754S and the double mutant C754–759S, 5′GACGATGGCGCCGAAAGCGGC; for C759S, 5′AGCGGCGCCGCTAAGATCTGA and

for C292S, 5′-GTGAAATCCTCATCTCAGGGC-3′. Single-stranded M13*nrd*A (Mao et al., 1989) was used as the template for all mutagenesis except for the double mutant C754–759S, for which M13*nrd*A containing the mutation C759S was used as the template. The resulting M13*nrd*A mutants were sequenced as described below, cloned into pT7-5 and transformed into *E. coli* K38 containing pGP1-2 (Tabor & Richardson, 1985) as previously described for the R1 mutant C225S (Mao et al., 1989). Strains K38/C230S, K38/C462S, K38/C462A, K38/C754S, K38/C759S, K38/C754–759S, K38/C439S, and K38/C292S were created.

DNA Sequencing. DNA sequencing was performed using the 2′,3′-dideoxy chain-termination method (Sanger et al., 1977) with Sequenase from United States Biochemicals and [α-^{35}S]dATPαS (specific activity 410 mCi/μmol) from Amersham. Oligonucleotide sequencing primers whose sequences were separated by intervals of approximately 150 base pairs along the *nrd*A gene were synthesized as described above for the mutagenesis primers. Single-stranded M13 containing the appropriate mutant genes were used as sequencing templates.

Growth and Induction of Mutants. All R1 subunit mutants were overproduced using a system developed for the expression of toxic genes (Tabor & Richardson, 1985) and used previously in this laboratory for the production of C225SR1 (Mao et al., 1989). The *E. coli* K38 cells employed for the overproduction were grown at 30 °C, induced at 42 °C for 0.5 h, and then kept at 37 °C for 3 h. These cells were then harvested by centrifugation, frozen in liquid N_2, and stored at –80 °C.

Isolation of RDPR Subunit R1. All steps in the isolation procedure were performed at 4 °C. Frozen cells (30–50 g) were resuspended in buffer (5 mL/g) containing 50 mM Tris–HCl (pH 7.6, 4 °C), 15 mM $Mg(OAc)_2$, 5% glycerol, and 4 mM DTT (buffer I). The cells were lysed by passage through a French pressure cell at 12 000–14 000 psi. Cell debris was removed by centrifugation at 17500*g* for 20 min at 4 °C. Streptomycin sulfate (5% in buffer I; 1:5 supernatant volume) was added to the supernatant with stirring over 15 min, followed by 15 min of additional stirring. The precipitate was removed by centrifugation at 17500*g* for 20 min. The supernatant was brought to 66% saturation in ammonium sulfate (39 g/100 mL) by addition, with stirring, over 20 min. After stirring for an additional 30 min, the precipitate was pelleted by centrifugation at 17500*g* for 20 min. The pellet was dissolved in buffer (1.2 mL/g of cells) containing 100 mM potassium phosphate (pH 7.6), 5% glycerol, and 4 mM DTT (buffer II), loaded onto a 5 × 53 cm column of Sephadex G-25, and eluted with buffer II. The brown-colored fractions were diluted with 1.5 volumes of buffer II and loaded slowly (flow rate 0.5 mL/min) onto a 3.5 × 38 cm dATP–Sepharose affinity column (Berglund & Eckstein, 1974). The loaded column was washed with buffer I until the A_{280nm} of the eluate was less than 0.1. R1 was eluted with buffer containing 50 mM Tris–HCl (pH 7.6, 4 °C), 15 mM $Mg(OAc)_2$, 5% glycerol, 10 mM DTT, and 10 mM ATP. Fractions containing protein (judged by A_{292nm}) were collected, pooled, diluted with buffer I, and loaded onto a 2.5 × 19 cm column of DEAE–Sepharose at a flow rate of approximately 0.5 mL/min. The protein was eluted with a 700 × 700 mL gradient of 0–400 mM sodium chloride in buffer I. Fractions containing protein (judged by A_{280nm}) and activity were pooled, concentrated to approximately 3 mL in an Amicon ultrafiltration apparatus employing a PM-30 membrane, and dialyzed for 5 h against 1 L of buffer containing 50 mM Tris–HCl (pH 7.6, 4 °C), 15 mM $Mg(OAc)_2$, 10 mM DTT, and 20% glycerol. The resulting protein solution was stored in 100-μL aliquots at –80 °C. A typical protein yield is 1 mg/g of cells.

CD Spectra. Circular dichroism spectra were recorded (at ambient temperature, 23 °C) as the average of five scans on a Jasco Model J500 spectropolarimeter using a 0.2-mm pathlength cell. Samples were prepared by diluting concentrated protein stock solutions with buffer containing 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, and 0.1 mM DTT. Protein concentrations, determined by UV absorption, were approximately 0.1 mg/mL.

RDPR Assay Using [U-^{14}C]-CDP. Dithiothreitol was removed from R1 by the procedure of Penefsky (1977). A typical assay solution contained, in a final volume of 420 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM [U-^{14}C]CDP (specific activity 5×10^5 cpm/μmol for wild-type enzyme and 2×10^6 cpm/μmol for mutant enzymes), 1 mM NADPH, 17 μM TR, 0.5 μM TRR, 1–2.5 μM R1 mutant, and 1 molar equiv of R2 per R1. Alternatively, 10 mM DTT replaced TR, TRR, and NADPH as reductant. An aliquot (100 μL) containing everything except R1 and R2 was taken as a zero time point. At various times subsequent to the addition of RDPR, a 100-μL aliquot was removed from the assay and quenched by a 1.5-min incubation in boiling water. After the aliquot was cooled on ice, 1 unit of *E. coli* alkaline phosphatase in 50 μL of 500 mM Tris–HCl (pH 8.5) was added, and the aliquot was incubated for 1 h at 37 °C. To each aliquot was then added 20 μL of carrier cytosine and deoxycytidine (80 nmol each), and the mixture was centrifuged for 1 min to pellet the denatured proteins. A portion of supernatant (160 μL) was loaded on a 1-mL Dowex 1–borate column (Steeper & Stewart, 1970) and eluted with 7 mL of water. A 1-mL portion of the effluent was subjected to scintillation counting, and the remainder was concentrated in vacuo to approximately 300 μL for HPLC analysis using an Alltech C_{18} column with water as eluate (flow rate 1 mL/min). The retention times for cytosine and deoxycytidine were 5 and 16.5 min, respectively. Fractions containing cytosine and deoxycytidine were pooled separately. The yield from the workup was determined by UV analysis, and products were quantified by scintillation counting. The specific activity of cytosine was assumed to be $^4/_9$ that of the starting CDP, since the cytosine contains only four of the nine carbons in the uniformly labeled substrate.

Single-Turnover Experiments with [U-^{14}C]-CDP. The wild-type or mutant R1 subunit was prereduced with 10 mM DTT for 20 min at 25 °C. DTT was subsequently removed by the procedure of Penefsky (1977). A typical assay solution contained, in a final volume of 100 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM [U-^{14}C]CDP [specific activity (3–6) × 10^6 cpm/μmol], 3–10 μM wild-type or mutant R1, and 1 molar equiv of R2. An aliquot containing everything except enzyme was used as a control. All components except enzyme were assembled in a septum-sealed vial and degassed by three cycles of freeze–pump–purge with argon–thaw. Degassed solutions were equilibrated at 25 °C, and the reaction was initiated by addition of enzyme. The reactions were allowed to proceed at 25 °C for various time periods from 30 s to 30 min and then were quenched by incubation in boiling water for 1.5 min. After the reaction mixture was cooled on ice, 50 μL of 500 mM Tris–HCl (pH 8.5) containing 1 unit of *E. coli* alkaline phosphatase was added. The solution was incubated at 37 °C for 1 h. The products were separated and quantified as described above.

Single-Turnover Experiments with [3′-^{3}H,U-^{14}C]UDP, C462SR1, and R2. C462SR1 was prereduced with 10 mM DTT for 20 min at 25 °C. DTT was removed by the procedure of Penefsky (1977). The assay contained, in a final volume of 1.5 mL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.25 mM TTP, 1 mM UDP (^{3}H, 3.05×10^6 cpm/μmol; ^{14}C, 7.30×10^5 cpm/μmol; $^3H/^{14}C = 4.18$), 20 μM C462SR1, and 20 μM R2 (with 0.8 equiv of tyrosyl radical per R2). All assay components except UDP were mixed, and the solution was equilibrated at 25 °C for 5 min. After the reaction was initiated by addition of [^{3}H,^{14}C]UDP, aliquots of the assay mixture were used to determine the time course of several processes as described in sections i–iii.

(i) To monitor tyrosyl radical loss and the increase in the A_{320nm} on the protein, 400 μL of assay solution was transferred to a quartz cuvette. The reaction was monitored spectrophotometrically from 200 to 800 nm for 1 h. The drop-line-corrected absorbance at 412 nm, $[A_{412nm} - (2A_{406nm} + 3A_{416nm})/5]$; $\epsilon = 1920$ M^{-1} cm^{-1} was used to determine the absolute tyrosyl radical concentration (Bollinger et al., 1991).

(ii) To monitor both product (uracil and dUDP) and volatile tritium production as a function of time, a 100-μL aliquot was removed from the assay mixture at reaction times of 0.5, 1, 2, 5, 10, 20, and 60 min and quenched by a 1-min incubation in boiling water. After the aliquot was cooled on ice, 50 μL of 0.5 M Tris–HCl (pH 8.5) containing 1 unit of *E. coli* alkaline phosphatase was added, and the solution was incubated at 37 °C for 1 h. Carrier solution (100 μL) containing 1 mM uracil and 1 mM 2′-deoxyuridine (dUrd) was added. The aliquot was centrifuged for 5 min to pellet the denatured proteins, the supernatant was removed, and the pellet was rinsed with 300 μL of water. The wash was combined with the supernatant, and the volatile ^{3}H was isolated by bulb-to-bulb distillation of the combined supernatants. Radioactivity in the distillate was determined by scintillation counting. The residue from the bulb-to-bulb distillation was redissolved in 1 mL of water and then concentrated in vacuo to approximately 300 μL for use in product analysis by reverse-phase HPLC on an ODS-II reverse-phase C_{18} column (Alltech) with water as eluate (flow rate 1 mL/min). Fractions of 1 mL were collected. Fractions containing uracil (retention time 10 min) and dUrd (retention time 35 min) were pooled separately. The resulting pools were analyzed by UV spectroscopy to determine percent recovery during workup and the products quantified by scintillation counting. The specific activity of the product uracil was taken to be $^4/_9$ that of the starting UDP, since only four of the nine carbon atoms present in the uniformly labeled starting UDP remain in uracil.

(iii) $^T(V/K)$ for the reaction of [3′-^{3}H,U-^{14}C]UDP with C462SR1·R2 was measured. The method for determining $^T(V/K)$ assumes that ^3H_2O production and uracil release are solely the result of chemistry catalyzed by the mutant R1. A selection effect on cleavage of the 3′ C–H bond of UDP by C462SR1 is, therefore, estimated as the ratio of the number of equivalents of uracil released to the total number of equivalents of tritium released (^3H_2O in addition to ^{3}H covalently bound to the protein). The amount of volatile tritium and uracil in the 60-min time point were determined as described above. To quantify the ^{3}H and ^{14}C bound to the protein, an additional 200-μL aliquot was taken at 60 min and analyzed using a Sephadex G-25 (1.5 × 20 cm) column equilibrated at 4 °C with 50 mM Hepes buffer (pH 7.6) containing 15 mM $MgSO_4$ and 1 mM EDTA. Fractions of 800 μL were collected, the fractions containing protein were pooled, and the amount of protein was quantitated by A_{280nm}. An aliquot of the same pool was then counted to quantify ^{3}H and ^{14}C label bound to the protein.

Kinetics of Interaction of R1 Mutants with CDP, and DTT as Reductant. Wild-type, C754S, C759S, and C754–759S RDPR R1s were assayed using stoichiometric R2, [U-^{14}C]-CDP, ATP, and varying concentrations of DTT as reductant. For each assay the appropriate R1 subunit was separated from DTT by the procedure of Penefsky (1977). The concentration of the DTT stock solution was determined using Ellman's reagent (Jocelyn, 1987).

For wt-R1 and C754SR1, a typical assay mixture contained, in a final volume of 100 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1.0 mM [U-^{14}C]CDP (specific activity 5.26×10^5 cpm/μmol), 2.3 μM wt-R1 or C754SR1, 2.3 μM R2, and 2–20 mM DTT. The assays were incubated at 25 °C for 10 min and then quenched by incubation for 1 min in a boiling water bath. A control reaction containing everything except enzyme was treated in the same manner. Additional control experiments ensured that the rate of dCDP production was linear for this time period at the greatest concentration of DTT used. The amount of dCDP in each time point was determined by the method of Steeper and Stewart (1970).

For the reaction mixtures containing C759SR1 and the double mutant C754–759SR1, aliquots were removed and analyzed at multiple times. Each assay contained, in a final volume of 320 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1.0 mM [U-^{14}C]CDP (specific activity 5.26×10^5 cpm/μmol), 1.2 μM mutant R1, 1.2 μM R2, and 0.5–80 mM DTT. The assay mixtures were incubated at 25 °C. Aliquots of 100 μL were removed at 2, 5, and 10 min after initiation and quenched by incubation in boiling water for 1 min. The amount of dCDP formed at each time point was determined as described above.

Assays using thioredoxin (67 μM), thioredoxin reductase (0.4 μM), and NADPH (1.0 mM) as reductant were identical to those described above except that DTT was omitted.

Preparation of R1 with an Additional SpeI Site. A silent unique *Spe*I site was introduced into the *nrd*A gene 14 bp downstream of the initiation codon by site-directed mutagenesis (as described above). The mutagenic primer used was CAGAATCTACTAGTGACAAAG, and the methods are described above.

Construction of Epitope Tagged nrdA (pET-R1) and Epitope Tagged Mutants. Epitope tagged R1 was constructed by ligating two short synthetic duplexes between the *Eco*R1 and *Spe*I sites of the site-directed mutant R1:*Spe*I. The R1:*Spe*I mutant contains a silent mutation (amino acids are not changed) which introduces a unique *Spe*I restriction site slightly downstream of the 5′ end of the *nrd*A gene. The nucleotide sequences AATTCAGGAGGTAAGTACTAT-GTACCCATACGACGT, CGTATGGGTACATAGTACT-TACCTCCTG, CCCAGACTACGCTAATCAGAATCTA, and CTAGTAGATTCTGATTAGCGTAGTCTGGGACGT were synthesized using a Biosearch 8600 DNA synthesizer. Subsequent to deblocking, the oligomers were purified on a 15% denaturing (7.5 M urea) polyacrylamide gel and eluted by the crush and soak method. The purified oligomers were phosphorylated separately in reactions containing, in a final volume of 30 μL, 100 μM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 70 mM DTT, 10 mM ATP, and 2 units of polynucleotide kinase. After 15 min at 37 °C, the reactions were heated to 70 °C to inactivate the enzyme. Complementary oligonucleotides were mixed in equal proportions and annealed by heating to 65 °C followed by slow cooling.

M13mp19 containing the mutant *nrd*A gene R1:*Spe*I was digested with *Spe*I and *Eco*R1, and the DNA was subjected to phenol/$CHCl_3$ extraction and ethanol precipitation. The DNA was dephosphorylated using calf intestinal alkaline phosphatase and the large *Eco*R1–*Spe*I fragment was isolated by standard procedures on a 0.7% low-melting agarose gel.

The vector (100 ng) and 4 pmol of each synthetic duplex were ligated in 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 50 μg/mL BSA, and 5 mM ATP in a total volume of 20 μL. The reaction was initiated by addition of 0.6 unit of T_4 DNA ligase, and the reaction mixture was incubated at 16 °C for 16.5 h. The ligation solutions were transformed into competent TG-1 cells. The plaques obtained were screened by restriction analysis. The appropriate piece of DNA was then subcloned into pT7-5 and transformed into K38 containing pGP1-2 as previously described for the R1 mutants. DNA sequencing of the above construct revealed that three epitope tags (two in the correct orientation, one in the incorrect orientation) were attached to the N-terminus of the gene for R1. However, isolation of the epitope tagged R1 protein and protein sequence analysis by automated Edman degradation revealed only the expected ET-R1.

Separation of wt-R1 from ET-C759SR1. ET-C759SR1 was loaded onto a MonoQ 5/5 column (Pharmacia) equilibrated with buffer containing 50 mM Tris–HCl (pH 8.0, 4 °C), 5% glycerol, 4 mM DTT, and 100 μM diethylenetriaminepentaacetic acid (DTPA). Protein was eluted using a discontinuous gradient of 0–220 mM NaCl (in the equilibration buffer) during the first 22 min and then 220–260 mM NaCl over the following 20 min. Fractions were pooled to form eight samples across the single protein peak and concentrated using Centricon 30s (Amicon). Each sample was assayed for reductase activity with the TR/TRR/NADPH reducing system using the spectrophotometric assay. Each sample was then assayed for reductase activity with DTT as reductant using the [^{14}C]CDP assay described above. Protein in each sample was determined using the method of Lowry et al. (1957). Finally, a control experiment measured recovery of wt-R1 activity subsequent to FPLC chromatography.

RESULTS

Induction and Isolation of Mutants of R1. Previous biochemical studies in conjunction with sequence alignments provided the rationale for the choice of mutant R1s to be prepared: C462S, C462A, C754S, C754S–C759S, and C230S (Lin et al., 1987; Nilsson et al., 1988). The proteins were overproduced by using a system which was developed by Tabor and Richardson (1985) for expression of toxic genes and previously used in this laboratory for the preparation of R1 mutants C225S and C759S. The mutant proteins, grown and induced as previously described (Mao et al., 1989), were isolated from K38 cells by using a dATP–Sepharose affinity column. The proteins were all judged to be greater than 90% homogeneous by SDS gel electrophoresis.

Structural Integrity of Mutants. Since no X-ray structural data on the R1 subunit exists, the structural integrity of the mutant R1s was investigated using several methods. To ensure the correct primary structure, the entire *nrd*A gene sequence was determined for wt-R1 and for the R1 mutants C462S, C462A, C759S, C754S, and C754–759S. The sequences of mutants C230S and C292S were confirmed only in the area within approximately 100 base pairs of the mutation. The sequences were found to be identical to the published *nrd*A sequence (Nilsson et al., 1988), except for the desired mutations and for bases 1578 (C) and 1579 (G). These latter two bases are in a region of the sequence found to be compressed when sequenced with the normal dGTP method. Use of dITP in place of dGTP suggests that the published sequence has these two bases reversed. Further support for this reassignment comes from N-terminal sequencing of peptides isolated subsequent to cyanogen bromide treatment of wt-R1 (Sjöberg et al., 1985). The reversal of bases 1578 and 1579 causes residues 526 and 527 of the protein sequence to change from asparagine and aspartate to lysine and histidine, respectively. One of the peptide sequences reported by Sjöberg (CNBr pool A, peptide I) contains residues 509–526. Residues 509–525 are precisely those predicted by the published sequence, but residue 526 is reported to be lysine rather than the asparagine predicted by the published sequence. Our sequencing data and Sjöberg's peptide sequence information therefore indicate that the primary sequences of our wild-type and mutant R1 subunits are correct.

Evidence was next sought to establish that the mutant proteins are properly folded. The CD spectra of wt-R1 and the R1 mutants C462S, C462A, C754S, C759S, and C754–759S were recorded (data not shown). The spectra of C462SR1 and C754SR1 precisely overlay that of the wt-R1, while the spectrum of C759SR1 shows slightly less ellipticity than that of the wild type, and the spectrum of C754–759SR1 shows slightly more ellipticity than that of wt-R1. These CD spectra suggest that the structures of the mutants are not drastically perturbed.

The possibility remains, however, that slight structural perturbations might inhibit substrate turnover by preventing the binding of substrate, allosteric effector, or the R2 subunit. The fact that all of the mutants were isolated with a dATP–Sepharose affinity column suggests that the allosteric effector binding site is unperturbed. Since the mutants C754S, C759S, and C754–759S are all capable of catalyzing the reduction of substrate (discussed subsequently), they must all be capable of binding both substrate and the R2 subunit. Since C462SR1 can mediate 3′ carbon–hydrogen bond cleavage of the substrate, this mutant must also be capable of binding both substrate and R2. Taken together, these results suggest that the gross structures of the mutant R1s are not significantly different from that of the wt-R1.

Characterization of C754S, C759S, and Double-Mutant C754S–C759S. Studies of Lin et al., (1987) and Mao et al. (1989) suggested that two cysteines (C754 and C759) might serve as redox shuttles between the active site thiols of R1 and the external reductant thioredoxin. This model predicts that R1s containing serine in place of either cysteine would be incapable of producing dCDP with thioredoxin. R1 mutants C754S, C759S, and C754S–C759S were therefore prepared and assayed for their ability to reduce CDP in the presence of a stoichiometric amount of R2, allosteric effector (ATP), and either the biological reducing system TR/TRR/NADPH or DTT. The results are summarized in Table I. The turnover numbers for dCDP production with thioredoxin are low, approximately 3% of that observed with the wt-R1. We have previously proposed with C759SR1 that the low levels of dCDP production are due to the fact that *E. coli* K 38 host cells in which these mutants are expressed contain a small amount of host cell wt-R1 (Mao et al., 1989). The relative amount of mutant R1 to wild type (or heterodimer of wild type and mutant) can be variable, depending on the efficiency of the heat induction in our expression system. As the conditions have been optimized for heat induction of the mutant proteins, rates of dCDP production have been minimized at approximately 3.0% of that observed with the wild-type enzyme and

Table I: Kinetic Parameters for dCDP Production by Wild-Type and Mutant R1s

	TR/TRR/NADPH		DTT	
enzymes	V_{max} (nmol min^{-1} mg^{-1})	K_m CDP (μM)	V_{max} (nmol min^{-1} mg^{-1})	K_m DTT (mM)
wt				
R1/R2 (1:1)[a]	660		118	20
R1/R2 (1:5)[a]	1310 (3300)[b]	73		
C754S				
R1/R2 (1:1)	41	129	61	11
C759S				
R1/R2 (1:1)	44	146	37	15
C759S–C754S R1/R2 (1:1)	32	166	439	3.7
C230S				
R1/R2 (1:1)[a,b]	480		48	
R1/R2 (1:5)[a,b]	1290		49	
C292S				
R1/R2 (1:1)[a,c]	410		35	
R1/R2 (1:5)[a,c]	560		63	

[a] The K_m value of R2 for R1 is 0.16 μM. While the K_d for subunit interaction is unknown, with wt-R1 an excess of subunit R2 is required to maximize activity. However, due to the reduced activity of the mutant proteins and hence the greater concentrations required for the assay, the molar ratio of R1:R2 does not seem to effect the activity in the same way as that observed with the wild-type enzyme. [b] The specific activity of wt-R1 has varied from 1300 to as high as 3300 with apparently homogeneous protein. The reasons for this variability with excess R2 are presently not understood. [c] The reactions were run with saturating CDP and a full kinetic analysis was not undertaken.

appear to be approximately the same for all of the mutants. Thus we interpret these data to indicate that this low level of dCDP production results from contaminating wt-R1 and that thioredoxin is not capable of reducing the active site thiols of the mutant R1s. This interpretation is supported by epitope tagging experiments described below, the results of our effort to separate mutant from contaminating wild-type protein.

When DTT was used as a reductant, dCDP production does occur. In fact, with C754SR1 and C759SR1, the K_m's and V_{max}'s are very similar to those observed with the wt-R1. The V_{max}'s for C754SR1 and C759SR1 are 50% and 30%, respectively, the rate of wt-R1. The similarity of the mutants to the wild type extends to the observation that at high concentrations of DTT (greater than 20 mM) inhibition of substrate reduction occurs with both wild-type and mutant R1s. An additional intriguing observation is that the double mutant C754S–C759SR1, which possesses background level activity with TR/TRR/NADPH, possesses activity with DTT almost identical to that observed with the wt-R1 and the TR/TRR/NADPH system. The reasons for this dramatic increase in activity are, at present, not understood. Several conclusions can be drawn from these results. The rapid rate of CDP reduction with this small organic molecule establishes that neither C754 nor C759 is directly involved in substrate reduction. The results with DTT and TR are consistent with the hypothesis that C754 and C759 are the cysteines that normally pass reducing equivalents from thioredoxin to the active site cysteines of R1 and that mutation of either impairs their normal function. Finally, the results with the double mutant and DTT suggest that DTT can bypass the C-terminal cysteines and reduce the active site cysteines directly.

To further investigate the catalytic capabilities of these mutants in comparison with the wt-R1, single-turnover experiments examining the rate and total amount of dCDP produced have been undertaken (Table II). If there is an active site for each of the two R1 promoters, then two dCDPs should be produced under single-turnover conditions.

Table II: Single-Turnover Experiments with Mutant R1s

R1	dCDPs/R1	R1	dCDPs/R1
wt	2.6 ± 0.3[a]	C759S	1.2 ± 0.1[c]
C462S	0.17	C759S–C754S	1.2 ± 0.1[d]
C754S	0.9 ± 0.1[b]		

[a] Average of 12 determinations. [b] Average of 11 determinations. [c] Average of six determinations. [d] Average of 10 determinations.

As indicated in Table II, prereduced wt-R1 in the absence of any external reductant can produce 2.6 equiv of dCDP. These results are similar to those we have previously reported (Lin et al., 1987). Accounting for greater than two dCDPs produced previously required us to postulate the redox shuttle model in which two cysteines are directly involved in substrate reduction and two cysteines shuttle electrons between the active site thiols and thioredoxin. While this model predicts the production of 4 equiv of dCDP, the substoichiometric number was rationalized in terms of an inability to adequately prereduce R1. If the shuttle cysteines (C754 or C759) were disabled and assuming incomplete prereduction of the mutant R1, 1.3 equiv of dCDP/R1 should be produced. This predicted number is half that observed for the wild-type enzyme since the putative redox shuttle has been disabled. This predicted value is similar to that observed for all three mutants. The rates of dCDP production by mutant R1s and wt-R1 were also investigated. In all cases, dCDP production was complete within the first 30 s. Measurements of the rate constants for reduction will therefore require rapid quench methodology.

Separation of wt-R1 from Mutant R1s Using Epitope Tagging. A number of methods have been attempted to remove contaminating wt-R1 from the mutant R1s. The one that has proven partially successful uses an epitope tagging method (ET) (Davis & Fink, 1990; Kolodziej & Young, 1989). The protocol involves attaching, to the N-terminus of R1, a peptide tail which would be recognized by a specific monoclonal antibody. An affinity column prepared from this antibody would then allow separation of the ET-mutant R1 from the wt-R1. The epitope YPYDVPDYA, recognized by the commercially available monoclonal antibody 12CA5, was added by genetic engineering to wt-R1 and to C759SR1. The method is described in detail under Materials and Methods.

The control ET-R1 had specific activity 1390 nmol min^{-1} mg^{-1} (R1:R2 = 1:5) with TR/TRR and 50 nmol min^{-1} mg^{-1} with 10 mM DTT, identical to that of wt-R1 without the additional amino acids. Western blot analysis revealed that the monoclonal antibody 12CA5 recognized the ET-R1 and not the wt-R1. However all attempts to selectively remove the native ET-R1 from the wt-R1 using the antibody or an antibody affinity column failed. It appears that the epitope is not readily accessible to the antibody under nondenaturing conditions.

While disappointed, we noticed that the ET-R1 (a dimer), would have four additional negative charges with respect to wt-R1. An attempt was therefore made to separate ET-R1 from wt-R1 using anion exchange, MonoQ, FPLC chromatography. The best separation was achieved at pH 8.0, in the absence of Mg^{2+}, using a discontinuous gradient of 0–260 mM NaCl (Figure 1A). Under these conditions, wt-R1 elutes at 22.5 min and ET-R1 elutes at 24.5 min. Sufficient resolution of these homodimers was achieved that we thought that the back side of the peak containing ET-mutant R1 would be free of wt-R1.

An epitope tagged version of C759SR1 was therefore prepared and analyzed for activity subsequent to chromatography by FPLC. The protein-containing fractions were

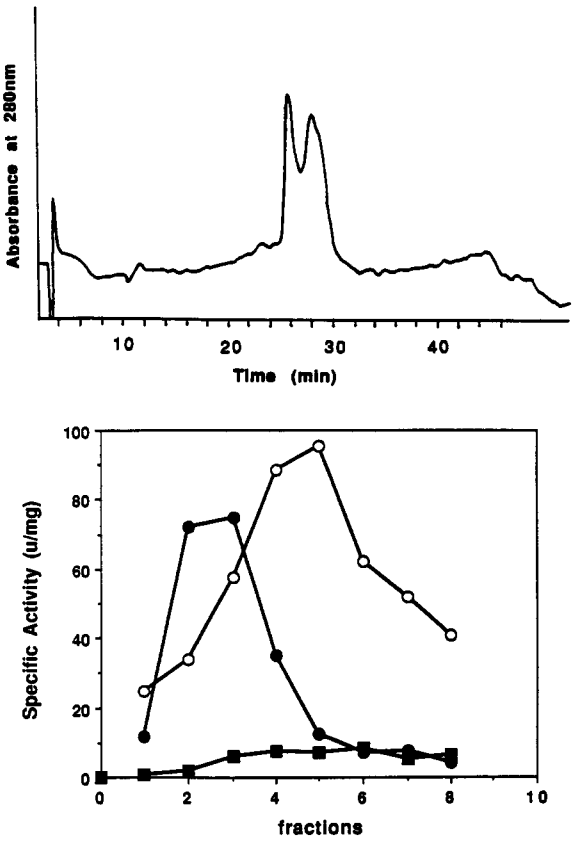


FIGURE 1: (A, top) Separation of wt-R1 and ET-R1 using MonoQ FPLC chromatography. The details of the gradient elution are described in Materials and Methods. (B, bottom) Chromatography of ET-C759SR1 on a FPLC MonoQ column. Assay for dCDP production: using TR/TRR/NADPH and the spectrophotometric assay (●); using DTT and the radioactive assay (■); protein (○). Specific activity of the protein prior to chromatography was 8 nmol min^{-1} mg^{-1} with DTT as reductant and 26 nmol min^{-1} mg^{-1} with TR/TRR/NADPH as reductant.

pooled to provide a total of eight samples that spanned the single protein peak (Figure 1B). Each fraction was assayed spectrophotometrically using TR/TRR/NADPH and radiometrically using DTT. The results indicate a clear peak of thioredoxin-dependent dCDP production that precedes the peak of protein. Note that the TR-dependent reductase specific activity is 3 times higher than that loaded onto the column. The activity with DTT as reductant, on the other hand, is essentially constant throughout the protein peak and is equal to the control sample ET-C759SR1 that had not been chromatographed (Figure 1B).

Given the activity elution profiles (Figure 1B) and the control protein elution profiles of ET-R1 and wt-R1 (Figure 1A), the simplest interpretation of the data is that the contaminant is *not* a homodimer of wt-R1, but rather a heterodimer of wt-R1 and ET-C759SR1. In retrospect, given the low concentrations (~3%) of contaminating wt-R1 based on activity, this result is expected. The anticipated total removal of the wt-R1 was therefore not achieved as the heterodimer elutes between the wt-R1 and the mutant R1. The specific activity of the C759SR1 determined from a pool of fractions 6–8 (Figure 1B) using TR/TRR/NADPH as reductant, in comparison with wt-R1 isolated by FPLC, now indicated that C759SR1 has 0.3% the activity of the wt-R1 with this reductant. This number is decreased by a factor of 10 from the activity prior to chromatography. On the other hand, the DTT-mediated reductase activity has remained unchanged by this chromatographic procedure. Attempts to make a new ET-R1 with additional negative charges to facilitate complete separation of mutant from wt-R1 have recently been successfully completed. Unfortunately, the specific activity of this newly prepared ET-protein, ET2-R1, was only 50% that of the wt-R1 and therefore the ET2-mutant R1s were not further analyzed. The present results, however, present additional support for the putative role of C759 as a redox shuttle between active site cysteines and thioredoxin.

Characterization of C462S and C462A as Active Site Reductants. Previous studies (Mao et al., 1989) reported the amazing observation that incubation of C225SR1, residue 225 being one of the active site cysteines thought to be directly involved in NDP reduction, with CDP and R2 resulted in destruction of the substrate, loss of the tyrosyl radical on R2, and cleavage of R1 into two polypeptides. In addition, cytosine release and an absorbance increase at 320 nm were detected, observations that are remarkably similar to our previous results with mechanism-based inhibitors 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP) (Ator & Stubbe, 1985) and 2′-azido-2′-deoxyuridine 5′-diphosphate (N_3UDP) (Salowe et al., 1987). Initiation of the complex chemistry observed with the nucleotide analogs and C225SR1 (see accompanying papers) has been shown to require 3′ C–H bond cleavage of the nucleotide.

If C462 is also crucial for the reduction reaction, then one might expect that a mutation to serine would still allow 3′ carbon–hydrogen bond cleavage, but that the lack of reducing ability would prevent dNDP production. As in the case of C225SR1, alternative chemistry might also be expected to occur. Incubation of C462SR1 with R2 and CDP results not only in production of low levels of dCDP, presumably due to contaminating wt-R1, but also in release of cytosine. The kinetics of these reactions using either the TR/TRR/NADPH or DTT reducing system are shown in Figures 2A and B. As previously reported for C225SR1 [Mao et al., (1989) and accompanying papers] the rate of cytosine release appears to be independent of the reductant (Figure 2B), consistent with the reaction being catalyzed by the mutant protein. The results contrast, however, with those previously reported with C225SR1 for dCDP production with the TR/TRR/NADPH reducing system. The C462SR1 is rapidly inactivated, producing 1.2 equiv of dCDP/C462SR1 (Figure 2A). On the other hand, with DTT as a reductant, steady dCDP production is observed. The rate is 13 nmol min^{-1} mg^{-1}, approximately equivalent to that expected for contaminating wt-R1 with this reductant. DTT, in addition to providing reducing equivalents, appears to be protecting the enzyme against inactivation.

These results led us to examine this reaction in more detail by using the available, isotopically labeled [3′-^{3}H,U-^{14}C]UDPs as substrate. Prereduced C462S-R1 and R2 were incubated with [^{3}H,^{14}C]UDP and allosteric effector TTP, and the reaction mixture was analyzed for ^3H_2O production, ^{3}H and ^{14}C covalently attached to the protein, change in absorption at 320 nm of the protein, loss of tyrosyl radical, uracil production, and cleavage of the R1 subunit into two polypeptides. A number of these results are shown in Figure 3. As expected, uracil is released and is accompanied by substoichiometric amounts of ^3H_2O. An apparent selection effect of 1.7 can be calculated from the ratio of equivalents of uracil produced to the equivalents of ^3H_2O and ^{3}H bound covalently to protein. In addition, 7 equiv of [^{14}C]sugar/equiv of C462SR1 was on the ^{3}H,^{14}C-labeled protein isolated by Sephadex G-25 chromatography from analysis of the 60-min time point.

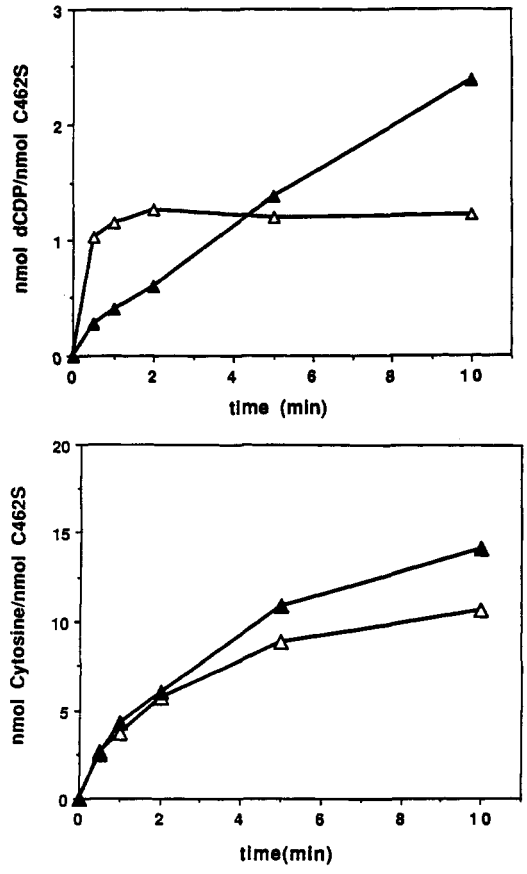


FIGURE 2: Incubation of C462SR1 with R2 and CDP. Analysis of the rate of product production: (A, top) dCDP formation using DTT as reductant (▲) and using TR/TRR/NADPH as reductant (△); (B, bottom) cytosine formation using DTT as reductant (▲) and using TR/TRR/NADPH as reductant (△).

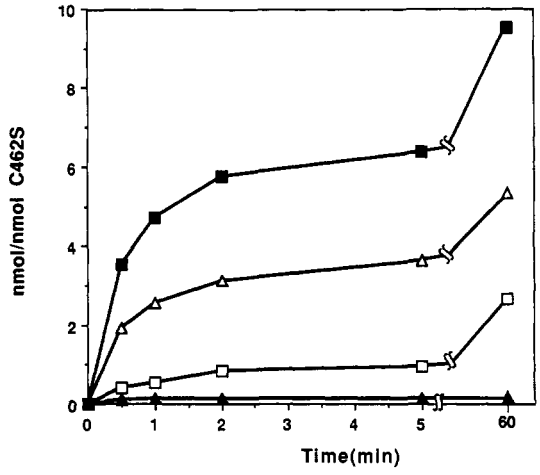


FIGURE 3: Rate of product production when C462SR1 is incubated with R2 and UDP in the absence of any external reductant: (■) uracil; (△) ^3H_2O; (□) A_{320nm} due to alkylation of the protein by 2-methylene-3(2*H*)-furanone; (▲) dUDP.

In analogy to the extensive investigations on the mechanism of ClUDP inactivation of RDPR, the observation of radiolabeled protein suggests that 2-methylene-3(2*H*)-furanone might be produced during this reaction. This intermediate can also be detected by a change in the absorption spectrum of the protein at 320 nm. As indicated in Figure 4, an absorbance change at 320 nm is, in fact, observed in addition to a tailing absorption from 340 to 420 nm. This type of tailing has previously been detected when protein is alkylated nonspecifically by several equivalents of 2-methylene-3(2*H*)-

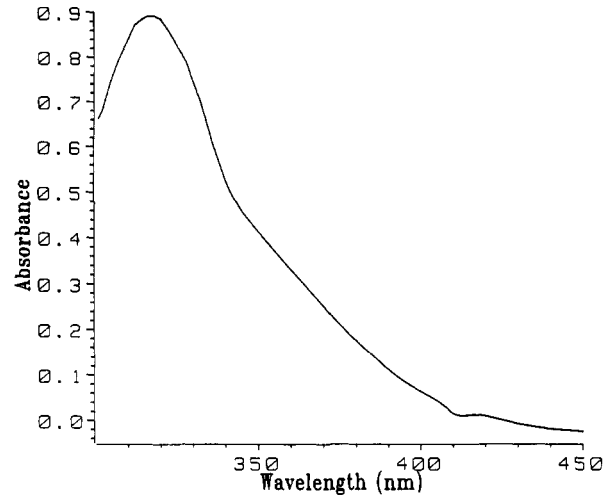


FIGURE 4: Absorption spectrum of C462SR1 subsequent to inactivation by CDP in the presence of R2. Displayed as a difference spectrum after subtraction of the protein spectrum at $T = 0$.

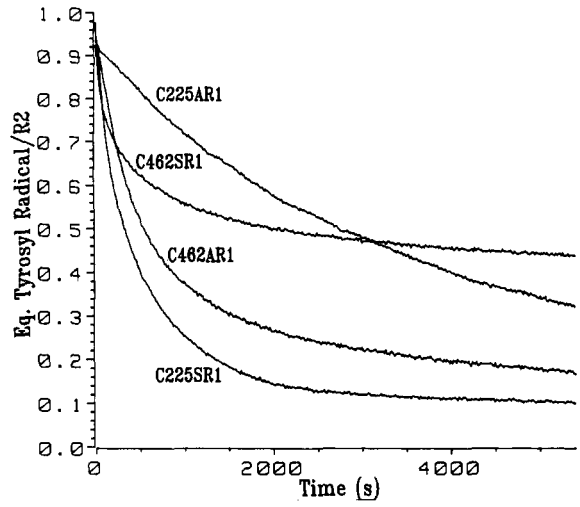


FIGURE 5: Loss of Y• on incubation of various mutant R1s with R2 and CDP.

furanone. The chemical structure(s) giving rise to absorption in this region has(have) not been identified. Assuming an extinction coefficient of 20 000 M^{-1} cm^{-1} for the species giving rise to the absorbance at 320 nm (Ashley et al., 1986), at the 60-min time point an upper limit of 2.7 equiv of chromophore/C462S is observed. (This number is in reality probably less than 2.0 as substantial absorption due to nonspecific alkylation is apparent in this region.)

As indicated in Figure 3, the kinetics of the interaction of UDP with C462SR1·R2 have also been examined. They are multiphasic. Within the first 5 min, 6.5 equiv of uracil and 3.8 equiv of ^3H_2O per equivalent of C462SR1 are produced. Over the next 55 min an additional 3.5 equiv of uracil and 1.6 equiv of ^3H_2O per equivalent of C462SR1 are liberated at a much slower rate. Even after 60 min the enzyme is not totally inactivated. Tyrosyl radical loss is also observed during the reaction. Its rate of disappearance correlates with uracil and ^3H_2O release (data not shown). There is a burst of tyrosyl radical loss followed by a much slower loss in absorbance at 410 nm. Inactivation of C462SR1 appears to be predominantly due to alkylation by the furanone. Finally, no cleavage of R1 into two polypeptides was observed under a variety of conditions.

C462SR1, R2, and UDP in dUDP Production. Also indicated in Figure 3 is that incubation of C462SR1, R2, and

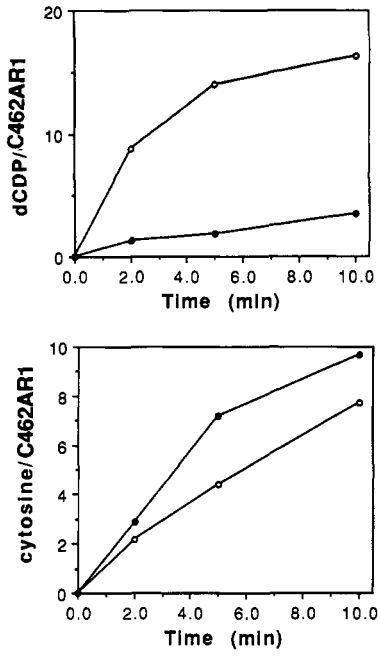


FIGURE 6: Incubation of C462AR1 with R2 and CDP. Analysis of the rate of product production using DTT (●) as reductant and using TR/TRR (○) as reductant. (A, top) Rate of dCDP production. (B, bottom) Rate of cytosine production.

UDP in the absence of external reductant results in the production of 0.1 equiv of dUDP. One would anticipate that only 0.078 dUDP could be produced per C462SR1 due to a 3% contamination with wt-R1. If C462 is indeed a redox-active thiol, then its replacement should prohibit all dUDP production. An explanation for the greater than expected amounts of dUDP will be provided in the accompanying papers (Mao et al., 1992a,b).

C462AR1, R2, and CDP. Recently Aberg et al. (1989) also employed site-directed mutagenesis to investigate the complex role of the redox-active thiols. These investigators reported that C462AR1 converts CDP to dCDP at 40% the rate of wt-R1 and interpreted their results to indicate that this cysteine is not in fact required for catalysis. A footnote in a recent paper by Larsen et al. (1992) states that this mutant is "catalytically inactive". Because their initial results were so different from those reported in this work with C462SR1, the C462AR1 was prepared and characterized. C462AR1 appears to behave in much the same way as the C462SR1. Interaction of the mutant with CDP results in catalytic production of cytosine that is independent of reductant, as observed with the serine mutant (Figure 6B). Cytosine release is accompanied by rapid loss of the tyrosyl radical (Figure 5), and alkylation of the R1 subunit by furanone is indicated by an absorption increase of the protein at 320 nm (data not shown). Inactivation, in contrast with the serine mutant, appears to be due to both loss of the tyrosyl radical and alkylation of R1 by the furanone. In addition, dCDP is produced at rates similar to those expected for contaminating wt-R1.

Examination of Additional Cysteine R1 Mutants. As control experiments, cysteine mutants of R1 C230S and C292S were prepared. Previous biochemical studies had suggested that C230S might be one of the cysteines directly involved in substrate reduction (Lin et al., 1987). C292 was chosen arbitrarily. Both of these mutants have activity comparable to that observed with the wt-R1 as indicated in Table I.

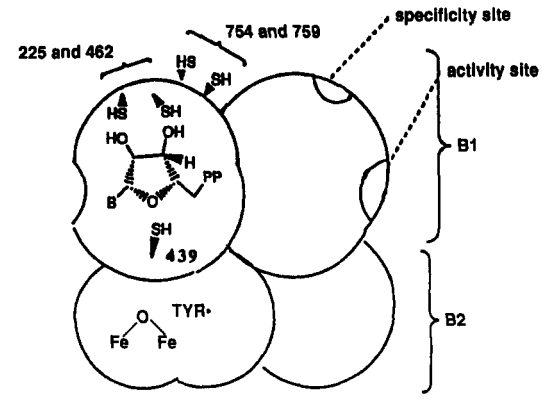


FIGURE 7: Model for the five cysteines involved in NDP reduction catalyzed by RDPR: postulated structure of RDPR from *E. coli*.

DISCUSSION

The studies reported in the present paper and accompanying papers in this issue allow us to put forth a new model for the role of the cysteines involved in RDPR-catalyzed nucleotide reduction (Figure 7). This model indicates that there are five cysteines on R1 required for catalysis. The model depicts the role of C754 and C759 as reductants of the active site cysteines C225 and C462, accepting electrons from thioredoxin and passing them into the active site disulfide resulting from reduction of NDP to dNDP. Several pieces of evidence support this function. The first is that mutation of either C754 or C759 to serine results in substantially reduced rates of dNDP production when TR/TRR/NADPH is used as a reductant, in the range of that expected for the contaminating wt-R1 activity present in all of our mutants. Efforts using an epitope tagging method designed to separate mutant from wt-R1 support this proposal as well. In contrast, when DTT is used as a reductant, dCDP is in fact produced at rates similar to that observed with wt-R1. Thus these cysteines are not required for nucleotide reduction. The single-turnover experiments also indicate that the number of dCDPs produced with the mutant R1s (C754S, C759S, or the double mutant) is approximately half that observed with the wt-R1. These results, although complex, again are consistent with a putative role for these thiols as electron shuttles. The rates of dCDP production with these mutants and wild-type enzyme, in the absence of external reductant, are all too fast to measure using conventional kinetics methods. These results contrast dramatically with the rates of dCDP production by C439S, C225S, and C462S mutants under similar conditions (see accompanying papers). These are the expected results if C754 and C759 are not directly involved in substrate reduction.

The role of these thiols is also supported by recent studies on the *Lactobacillus leichmannii* reductase (RTPR). This enzyme utilizes adenosylcobalamin as a cofactor and nucleoside triphosphates as substrates (Lin et al., 1987). Attempts to identify the cysteine residues oxidized concomitantly with substrate reduction, as in the case of the *E. coli* RDPR, revealed the involvement of multiple cysteines. Unexpectedly, one peptide from RTPR identified by these studies displays sequence identity with the C-terminal region of *E. coli* R1 subunit. This region contains C754 and C759. Since the *E. coli* thioredoxin is capable of functioning as a reductant for the *L. leichmannii* reductase, these data are consistent with the role of C754 and C759 as conduits of reducing equivalents.

The model in Figure 7 also proposes that C225 and C462 are directly involved in substrate reduction. Mutagenesis of one of these cysteines should uncouple the postulated first step in this reaction sequence, 3′ carbon–hydrogen bond

cleavage, from the actual reduction process. Such an uncoupling was previously observed with mechanism-based inhibitors, ClUDP and N_3UDP [Ator and Stubbe (1985); Salowe et al., unpublished results]. Interaction of both of these substrate analogs with wt-R1 subsequent to 3′ carbon–hydrogen bond cleavage of the nucleotide resulted in glycosidic bond cleavage, elimination of PP_i, production of 3-methylene-3(2*H*)-furanone, and enzyme inactivation. In the case of the N_3UDP, inactivation was also accompanied by loss of the tyrosyl radical on R2. Results reported in this and an accompanying paper suggest that similar chemistry occurs with the normal substrate and two mutant R1s (C462S and C225S).

The observation of ^3H_2O release when [3′-^{3}H,U-^{14}C]UDP is incubated with C462SR1·R2 indicates that 3′ carbon–hydrogen bond cleavage has occurred and ensures that this mutant's active site remains intact. However, this mutation appears to impair the ability of this mutant R1 to act as a reductant. As indicated in Figures 2A and 3, incubation of an NDP with C462SR1·R2 and the TR/TRR/NADPH reducing system results in rapid inactivation of dNDP formation. These results contrast with the slower but continuous rate of dNDP formation in the presence of reductant DTT or the continuous release of uracil (cytosine) regardless of the reductant (Figure 2A).

These observations are consistent with enzyme-mediated conversion of the normal substrate into the alkylating agent 2-methylene-3(2*H*)-furanone. This compound was previously identified as being responsible for inactivation of RDPR by ClUDP and N_3UDP. The kinetic results can be explained if this furanone which accompanies uracil release in the first few minutes of the reaction modifies a residue(s) near the active site of both the wild-type and mutant R1s giving rise to the 320-nm absorbance change on the protein (Figure 4). The rapid loss in ability to produce dCDP can be rationalized as the result of inactivation of the contaminating wt-R1 (Figure 2A). The furanone could alkylate the surface thiols, C754 or C759. This modification would prevent reduction of CDP to dCDP by wt-R1 due to the inability of thioredoxin to mediate rereduction of the active site thiols. DTT, on the other hand, could prevent this rapid inactivation by trapping the furanone, allowing continued production of dCDP. A similar alkylation of the mutant protein and perhaps the wt-R1 could, however, continue to allow NDP binding and 3′ carbon–hydrogen bond cleavage, resulting in cytosine release. Subsequent to this covalent modification, the kinetics of cytosine release might be expected to be altered due to altered binding of substrate NDP. This could explain the second slow phase of the reactions observed experimentally (Figure 3).

Quantitation of this inactivation process is also similar to previous studies on the inactivation of RDPR by ClUDP. Analysis of the 60-min time point revealed seven furanones covalently bound to R1, while quantitation of the extent of alkylation by absorbance at 320 nm revealed approximately two furanones bound. This apparent discrepancy can be easily rationalized with the realization that the furanone is a bifunctional alkylating agent. Reaction of the more reactive exocyclic methylene yields a modified protein with a λ_{max} at 260 nm masked by the protein absorption. Five furanones are presumably bound in this fashion. However, modification at both the exocyclic methylene and the C-1 carbon, the latter by an amino group, was previously proposed to give rise to the 320-nm absorption (Ashley et al., 1986). Two furanones appear to be bound in this manner.

In the case of C462SR1, a single conservative substitution has resulted in the conversion of the normal NDP substrate into a mechanism-based inhibitor. A similar observation has been made with C225SR1 [Mao et al. (1989) and accompanying papers]. The fact that the observed chemistry is remarkably similar to studies with the mechanism-based inhibitor ClUDP and that this well-characterized chemistry occurs when the RDPR is in the oxidized as well as reduced state suggests that reducing equivalents are *not* required for the chemistry observed with C462S and C225S R1s. These results provide the basis for the hypothesis that C462 and C225 are involved in the direct reduction of the substrate.

Finally, an essential feature of the new model (Figure 7) is that C439 is the protein radical required for 3′ carbon–hydrogen bond cleavage of the substrate. The model proposes that, in the active ternary complex (R1·R2·NDP), a protein radical on R1 is generated by long-range electron transfer to the tyrosyl radical on R2. This model predicts that C439 is essential, and evidence to support this contention is provided in an accompanying paper. Several questions concerning the chemical feasibility of this process are immediately posed by such a hypothesis. (1) Are the redox properties of a tyrosyl radical on the R2 subunit such that it can oxidize cysteine 439 to a thiyl radical on the R1 subunit? (2) How might such an electron transfer occur? (3) Can a thiyl radical mediate hydrogen atom abstraction from a 3′ carbon–hydrogen bond of the nucleotide? Each of these questions will briefly be discussed in turn.

The ability of a tyrosyl radical to generate a thiyl radical has precedent in the recent work of Rao et al. (1990). Using rapid-flow EPR spectroscopy they have shown that an acetaminophen radical generated by horseradish peroxidase is reduced by glutathione to form a thiyl radical of glutathione, observed as its disulfide radical anion. Thus the reduction potentials of a tyrosyl radical and a thiolate might be subtly tuned to mediate electron transfer.

The second question involves the mechanism by which a thiyl radical can be generated on R1. The recent X-ray structure of the R2 subunit of RDPR (Nordlund et al., 1990) provides both constraints and insight into this mechanism. The tyrosyl radical on R2 is buried and is ~10 Å from the nearest surface. It is thus unlikely that the Y• is directly involved in substrate reduction as the substrate binds to R1. Generation of a thiyl radical on R1, barring large conformational changes, thus requires that the electron transfer occurs over a distance greater than 10 Å. A comparison of a number of sequences of R2 reveals that there are relatively few conserved residues in this subunit other than the ones directly involved in coordination of the dinuclear iron center and the tyrosyl radical binding pocket (Nilsson et al., 1988). One such conserved residue is W48 and a second is Y356. The former, located on the surface nearest to the tyrosyl radical, has been proposed by Nördlund et al. to be a conduit to R1. The latter is found at the C-terminal end of R2, which is not observed in the X-ray structure (presumably due to thermal lability). Data of Sjöberg and her collaborators demonstrate that this region of R2 is responsible for interaction with R1 (Climent et al., 1991). Recent site-directed mutagenesis studies of Sjöberg and co-workers suggest that this residue might function to transfer electrons to Y122 from the R1 subunit (Climent et al., 1992). Electron-transfer reactions in which the electrons are transferred 20 Å with rate constants of 100 s^{-1}, greater than the observed turnover number of RDPR of 13 s^{-1}, have been reported (Bowler et al., 1990). Thus

transfer of electrons between R1 and R2 is both structurally and kinetically feasible.

The final question addresses the ability of a thiyl radical to mediate hydrogen atom abstraction. Thiols are excellent radical scavengers, rapidly donating a hydrogen atom to carbon-centered radicals. Thus, this step appears to be unappealing from a chemical point of view. Pulse radiolysis studies of Akhlaq et al. (1987) have shown, however, that thiyl radicals generated via hydrogen atom abstraction by hydroxyl radical are able to convert *cis*-2,5-dimethyltetrahydrofuran (**1**) to its trans isomer **2**.

1 2

The rate constants for hydrogen atom abstraction are small $\sim 10^3$ M^{-1} s^{-1}, in comparison with their ability for hydrogen atom donation (10^8 M^{-1} s^{-1}), and hence such processes are kinetically invisible. However, if hydrogen atom abstraction is followed by a rapid irreversible step (for example, loss of H_2O), then the unfavorable reaction should be driven to the right (Huyser, 1969). Also of interest are the reported homolytic bond dissociation energies of 91 kcal/mol for S–H (Griller & Kanabas-Kaminska, 1988) and 94 kcal/mol for *H–C–O* (Akhlaq et al., 1987) making this hydrogen atom abstraction thermodynamically feasible.

Based on the above chemical precedent, the model shown in Figure 7 is chemically viable. It should be emphasized that Figure 7 presents a hypothesis which at present is consistent with all of the available experimental information. Whether the R2 subunit simply functions as a cofactor to mediate production of a protein radical on R1, in much the same way that we have postulated that adenosylcobalamin functions to generate a protein radical in the *L. leichmannii* reductase, is the subject of intense investigation.

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Registry No. CDP, 63-38-7; DTT, 3483-12-3; RDPR, 9047-64-7; cysteine, 52-90-4.