Characterization of C439SR1, a Mutant of *Escherichia coli* Ribonucleotide Diphosphate Reductase: Evidence That C439 Is a Residue Essential for Nucleotide Reduction and C439SR1 Is a Protein Possessing Novel Thioredoxin-like Activity

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ABSTRACT: Ribonucleotide reductase from *Escherichia coli* catalyzes the conversion of nucleotides to deoxynucleotides. Cysteine 439 is proposed to be the protein radical on R1 which initiates the reduction reaction by cleavage of the 3′ carbon–hydrogen bond of the nucleotide (Mao et al., 1992a,b). C439 is thus proposed to be essential for catalysis. The C439S mutant of R1 (C439SR1) was prepared. The structure of this mutant was determined to be similar to wt-R1, based on identical CD spectra, isolation via an affinity column specific for the allosteric binding domain, binding of the substrate GDP, and competition with R1 for binding to R2. Preparations of C439SR1 are contaminated with low levels of wt-R1 due to the expression system. The wt-R1 in these preparations can be specifically inactivated by the stoichiometric mechanism-based inhibitor, 2′-azido-2′-deoxyuridine 5′-diphosphate. The activity of the resulting C439SR1 was shown to be less than 0.03% that of the corresponding wt-R1. This is the lower limit of detection with the present assay method. Thus C439 appears to be essential for catalysis. During these studies an unexpected activity of the C439SR1 was uncovered. Its additional cysteines, presumably C754 and C759, appear to function as a thioredoxin with the wt-R1, even though it is incapacitated with respect to nucleotide reduction.

Ribonucleotide diphosphate reductases (RDPRs) catalyze the conversion of nucleotides to deoxynucleotides, playing an essential role in DNA biosynthesis (Thelander & Reichard, 1979; Stubbe, 1990; Eriksson & Sjöberg, 1989). The protein requires two subunits for activity, R1 and R2, each composed of two identical polypeptide chains. As indicated in the two preceding papers (Mao et al., 1992a,b), R1 is proposed to be the business end of the protein, containing the substrate and allosteric effector binding sites and the five cysteines thought to be required for nucleotide reduction. The R2 subunit, which contains the unusual tyrosyl radical–dinuclear iron center cofactor, is proposed to initiate catalysis by long-range electron transfer. Reduction of the tyrosyl radical is proposed to generate a C439 radical required to initiate 3′ carbon–hydrogen bond cleavage of the NDP substrate. Given this postulated role for C439, replacement of this residue with a nonredox-active amino acid residue of the same size, such as serine, should provide a totally inactive R1 incapable of producing dCDP. These results should contrast with the C225SR1 and C462SR1 mutants, in which CDP is converted into cytosine (Mao et al., 1989, 1992a,b), and with C754SR1 and C759SR1 mutants, which produce dCDP in the presence of DTT (Mao et al., 1992a,b), but not with the normal reductant thioredoxin.

C439SR1 was therefore prepared and its ability to produce dCDP assessed. Studies using a mechanism-based inhibitor N_3UDP [Salowe et al. (1987), Thelander et al. (1976), Sjöberg et al. (1983), and Stubbe et al., unpublished data] to stoichiometrically inactivate wt-R1 present in all of the C439SR1 preparations suggest that C439 is essential for catalysis. During these studies an additional unanticipated catalytic role of C439SR1 was uncovered. This protein can function as a thioredoxin with wt-R1 allowing multiple turnovers (dCDP) albeit at very slow rates. The results of experiments justifying these conclusions are the subject of the present paper.

MATERIALS AND METHODS

Escherichia coli C439R1 was isolated from overproducing strain K38/C439SR1 (ϵ_{280nm} = 189 000 M^{-1} cm^{-1}) as previously described for C225SR1 (Mao et al., 1989). Subunit R2 was isolated from strain N6405/pSPS2 [ϵ_{280nm} = 130 500 M^{-1} cm^{-1}; specific activity 8000 nmol mg^{-1} min^{-1} (Salowe et al., 1986)]. Thioredoxin (TR) was isolated from strain SK3981 [specific activity 36 units mg^{-1} (Lunn et a., 1984)]. Thioredoxin reductase (TRR) was isolated from K91/pMR14 [specific activity 1000 units/mg (Russel & Model, 1985)]. *E. coli* alkaline phosphatase and thymidine triphosphate (TTP) were purchased from Sigma. [U-^{14}C]Cytidine 5′-diphosphate (CDP; specific activity 532 mCi/mmol), [5′,8-^{3}H]guanosine diphosphate (GDP; specific activity 9.0 Ci/mmol), and [^{32}P]-phosphate (specific activity 8500–9100 mCi/mmol) were purchased from New England Nuclear. [U-^{14}C]CDP and [5′,8-^{3}H]GDP were prepared by diluting with unlabeled compound and used without further purification. Dithiothreitol (DTT) was purchased from United States Biochemical. Ultrafree-MC filter units with polysulfone PTTK membranes, molecular weight cutoff 30 000, were obtained from Millipore. [β-^{32}P]-2′-Azido-2′-deoxyuridine diphosphate (N_3UDP) was prepared by the method of Salowe et al. (1987). All other materials were purchased in the highest purity available.

UV/visible absorption 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. SDS/PAGE was performed as described by Laemmli (1970). HPLC chromatography was performed on an Altex system with a Hitachi 100-40 detector.

Preparation of C439SR1. Site-directed mutagenesis was performed by the method of Taylor et al. (1985) using a kit purchased from Amersham. An oligonucleotide primer for preparation of C439SR1, 5′-TCTAACCTGTCTCTGGAGA-

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TA-3', was synthesized using a Biosearch 8600 (Milligen Biosearch, Millipore) DNA synthesizer and purified by use of a 20% PAGE/TBE gel (Mantiatis, et al., 1982). The mutant gene was sequenced using the 2',3'-dideoxynucleotide chain terminated method (Sanger et al., 1977) with Sequenase from United States Biochemicals and [α-^{35}S]-dATPαS (specific activity 410 mCi/μmol) from Amersham. The resulting mutant was cloned into pT7-5 and transformed into *E. coli* K38 containing pGP1-2 (Tabor & Richardson, 1985) as previously described for preparation of C225SR1 (Mao et al., 1989). C439SR1 was purified by the procedure of Mao et al. (1992a), and the protein purity was judged to be greater than 90% using 7.5% SDS/PAGE electrophoresis.

CD Measurement. Circular dichroism spectra were recorded as the average of five scans on a Jasco Model J500 spectropolarimeter using a 0.2-mm path-length cell at ambient temperature (23 °C). A typical sample contained 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.1 mM DTT, and 0.35 μM C439SR1.

Binding Assay of C439SR1 with [5',8-^{3}H]GDP. The K_d for GDP binding to C439SR1 was determined by the method of Ormö and Sjöberg (1991). C439SR1 was prereduced in 10 mM DTT at 25 °C for 20 min. A typical assay solution, in a final volume of 100 μL, contained 50 mM Tris–HCl (pH 7.6), 15 mM $MgSO_4$, 0.05 mM TTP, 2 mM DTT, 20 μM C439SR1, and various concentrations of [5',8-^{3}H]GDP (25–400 μM) (specific activity 1.4×10^8cpm/μmol). Each assay solution was equilibrated at 25 °C for 5 min. A 20-μL aliquot was removed and subjected to scintillation counting to obtain the total amount of GDP present. The remainder of the solution was centrifuged at 3000*g* through an Ultrafree-MC 30K filtration unit (Millipore) at 25 °C for 1 min. A 20-μL aliquot of the filtrate (usually 30–35 μL) was counted to quantify the amount of free GDP (L). The amount of bound GDP was calculated as the difference between the total GDP and the unbound GDP. The binding ratio (ν, nmol of GDP/nmol of C439SR1) was calculated from a M_r = 171 kDa for R1 and an extinction coefficient at 280 nm of 180 000 M^{-1} cm^{-1}. Each 20 μL of assay solution contained 0.4 nmol of C439SR1. A Scatchard plot was used to obtain both total number of binding sites (n) and dissociation constant(s) (K_d) from the equation $\nu = n - K_d(\nu/[L])$. Attempts to do similar experiments with CDP as substrate failed due to the high K_d.

C439SR1 as a Competitive Inhibitor of wt-R1's Interaction with R2. A typical assay solution contained the following: 100 μM CDP, 1.45 mM ATP, 0.18 mM NADPH, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 24 μM TR, 0.10 μM TRR, 0.1 μM R2, variable amounts of wt-R1 (0.06, 0.10, 0.20, 1.0 μM), and variable amounts of mutant R1 (0, 1.5, 3.0, 5.0 μM). Since C439SR1 contains contaminating wt-R1 activity, a control lacking wt-R1 was run for each C439SR1 concentration used. This value was then subtracted from the assays at each defined concentration of C439SR1.

Assay of Mutant C439SR1. Dithiothreitol (DTT) 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 2.33×10^6 cpm/μmol), 1 mM NADPH, 17–67 μM TR, 0.5 μM TRR, 1–2.5 μM C439SR1, and 1 molar equiv of R2 or R1. Alternatively, 10 mM DTT replaced NADPH/TR/TRR as reductant. A 100-μL aliquot contained everything except C439SR1, and R2 was taken as a zero time point. When CDP of high specific activity was used, small amounts of cytosine were routinely observed. This control was therefore routinely run. At various times, subsequent to addition of R1 and R2, a 100-μL aliquot was removed from the assay mixture and quenched by a 1.5-min incubation in a boiling water bath. 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. The aliquot was incubated at 37 °C for 1 h. To each aliquot, 20 μL of carrier cytosine and deoxycytidine (80 nmol) were added and the mixture was centrifuged for 1 min to pellet the denatured protein. A portion of supernatant (160 μL) was analyzed using Dowex 1–borate columns (5 mL) and eluted with 7 mL of H_2O (Steeper & Stuart, 1970). A 1-mL portion of the eluate was subjected to scintillation counting and the remainder concentrated in vacuo for further analysis by reverse-phase HPLC. A C_{18} column (Alltech) was used with water as eluate (flow rate 1 mL/min); the retention times for cytosine and deoxycytidine were 5 and 16.5 min, respectively. The fractions of cytosine and deoxycytidine were pooled separately. Percent recovery was determined by UV analysis, and the products were quantified by scintillation counting.

Single-Turnover Experiments with [U-^{14}C]CDP and C439SR1. C439SR1 was prereduced with 10 mM DTT at 25 °C for 20 min, and DTT was removed by the procedure described above. A typical single-turnover assay solution contained, in a final volume of 320 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.5 mM [U-^{14}C]CDP (specific activity 7.6×10^6 cpm/μmol), 0.25 mM TTP, 18 μM C439SR1, and 18 μM R2. The assay solution minus R1 and R2 was equilibrated at 25 °C, and these subunits were added to initiate the reaction. At various times, a 50-μL aliquot was removed, quenched, and analyzed using the Dowex 1-borate columns described above. In the control experiment, 50 μL of same assay solution in the absence of C439SR1 and R2 was analyzed to determine the background of contaminating cytosine.

Thioredoxin-like Activity of C754 and C759 of C439SR1: Assay of wt-R1 Using N_3UDP Inactivated C439SR1 as Reductant

(1) Inactivation of C439SR1 with N_3UDP. C439SR1 was prereduced with DTT, which was then removed as described above. The inactivation solution contained, in a final volume of 200 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1 mM N_3UDP, 0.25 mM TTP, 27 μM C439SR1, and 27 μM R2. The reaction mixture was incubated at 25 °C for 30 min. N_3UDP was removed by the procedure of Penefsky (1977). The inactivated C439SR1 solution (solution A) was diluted to a final volume of 340 μL containing 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.25 mM TTP, 0.5 mM [U-^{14}C]CDP (specific activity 7.6×10^6 cpm/μmol), 13 μM inactivated C439SR1, and 13 μM R2. This solution was incubated at 25 °C. A 40-μL aliquot containing 0.5 nmol of C439SR1 was removed at 0 and 120 min, quenched, and analyzed for product formation by standard procedures.

(2) Oxidation of wt-R1. Wild-type R1 was prereduced with DTT, which was removed as described above. Assay solution B contained, in a final volume of 380 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 0.5 mM [U-^{14}C]CDP (specific activity 7.6×10^6 cpm/μmol), 0.25 mM TTP, 6 μM R1, and 6 μM R2. The solution except R1 was equilibrated at 25 °C. Subunit R1 was added to initiate the reaction. At various times, a 50-μL aliquot containing 0.03 nmol of wt-R1 was removed, quenched, and analyzed as described above. At time 30 min, a 20-μL aliquot of solution B was used to investigate the thioredoxin-like activity of inactivated C439SR1 described above.

(3) *Turnover of wt-R1 Catalyzed by N_3UDP-Inactivated C439SR1.* To 20 μL of oxidized wt-R1 (solution B) was added 260 μL of solution A (containing inactivated C439SR1). The Final concentrations of C439SR1 and oxidized wt-R1 were 12 and 0.4 μM, respectively. At various times a 50-μL aliquot containing 0.60 nmol of C439SR1 and 0.02 nmol of wt-R1 was removed, quenched, and analyzed to determine the production of dCDP. A control reaction was run under identical conditions in which 20 μL of solution B contained everything except wt-R1. Solution A (260 μL) was added to solution B and analyzed as described for the experiment.

Incubation of C439SR1 with [β-^{32}P]N_3UDP: Inactivation of the Contaminating wt-R1 in the C439SR1 Preparations and Establishment of the Essentiality of C439

(1) *Inactivation of C439SR1 with N_3UDP.* The inactivation solution contained, in a final volume of 175 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 5.5 mM NaF, 0.20 mM TTP, 15 μM TR, 0.5 μM TRR, 0.7 mM NADPH, 1.4 mM N_3UDP, 7 μM C439SR1, and 7 μM R2. In the control experiment water replaced the enzymes. At various times a 20-μL aliquot was removed and assayed for activity by diluting into an 80-μL assay solution containing 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 16 μM TR, 0.6 μM TRR, 1 mM NADPH, 1 mM [U-^{14}C]CDP (specific activity 7.56 × 10^{6}cpm/μmol). Each assay was incubated at 25 °C for 10 min, quenched, and analyzed with Dowex 1–borate columns as described above.

(2) *Quantitation of Pyrophosphate Released Accompanying Inactivation of C439R1.* The inactivation solution contained, in a final volume of 240 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 8 mM NaF, 0.25 mM TTP, 50 μM [β-^{32}P]N_3UDP (specific activity 3.5 × 10^8 cpm/μmol), 10 μM C439SR1, and 10 μM R2. In the control experiment, 10 μM metR2 (the Y• reduced in 50 mM hydroxyurea for 30 min at 25 °C) was used in place of R2. After equilibration at 25 °C, the inactivation was initiated by addition of N_3-UDP.

A 100-μL aliquot containing 1 nmol of C439SR1 was removed to analyze for pyrophosphate production after 30 min. A freshly prepared tetrasodium pyrophosphate carrier solution (100 μL, 10 μmol) was added to this aliquot, which was diluted with 3 mL of 75 mM ammonium acetate (pH 5.0). The solution was extracted with acid-washed charcoal (1 × 250 mg, 2 × 100 mg) to remove the unreacted N_3UDP. The solution was then diluted with 10 mL of water, adjusted to pH 7.0 with 1 N NaOH, and chromatographed on a DEAE–Sephadex A-25 column (4 mL). A linear gradient (60 × 60 mL) of 0–600 mM triethylammonium bicarbonate (pH 7.5) (TEAB) was used to separate the pyrophosphate product from the breakdown product inorganic phosphate. Fractions containing phosphate (250 mM TEAB) and pyrophosphate (400 mM TEAB) were pooled separately. The amount of P_i recovered in the experiment and in the control was identical. A phosphate assay (Ames & Durbin, 1960) was used to determine the recovery of pyrophosphate in both the experiment and the control. A portion of the pyrophosphate-containing solution from both the experimental and the control was quantitated using scintillation counting. The amount of [^{32}P]PP_i released from the reaction mixture was obtained by subtraction of the radioactivity in the control experiment containing met R2.

To assay the amount of activity remaining at various times, a 20-μL aliquot of the inactivation solution was assayed in a final volume of 100 μL containing 50 mM Hepes (pH 7.5), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM NADPH, 25 μM TR, 0.8 μM TRR, 1 mM [U-^{14}C]CDP (specific activity 3.65 × 10^{6}cpm/μmol) at 25 °C for 20 min. Specific activities were determined by the production of dCDP using Dowex 1–borate columns as described above.

(3) *Characterization of the PP_i Isolated by Anion Exchange Chromatography: Conversion of Pyrophosphate to Phosphate.* An assay solution contained, in a final volume of 500 μL, 50 mM Hepes (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 8 mM NaF, 0.25 mM TTP, 50 μM [β-^{32}P]N_3UDP (specific activty 2.01 × 10^{8}cmp/μmol), 12 μM C439SR1, and 10 μM R2. In the control experiment, 10 μM metR2 (Y• reduced in 50 mM hydroxyurea for 30 min at 25 °C) was used in place of R2. The reaction mixture was incubated at 25 °C for 60 min. Carrier pyrophosphate (1 μmol) was added to the reaction mixture, and the workup proceeded as described above. The pyrophosphate pool (specific activity 1.85 × 10^5 cpm/μmol) from this large-scale reaction mixture was concentrated to dryness in vacuo to remove TEAB. The residue was redissolved in 20 μL of water. A portion of the concentrated pyrophosphate solution (containing 1900 cpm) was incubated with 4 units of yeast pyrophosphatase (Sigma) in a final volume of 25 μL containing 100 mM Tris–HCl (pH 7.2), 0.2 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) at ambient temperature for 1 min. The reaction was quenched by a 1-min incubation in a boiling water bath. The reaction mixture was analyzed on a PEI-cellulose TLC plate (Merck) prerun with H_2O. The elution solvent was 0.5 M $KHSO_3$ as previously described (Harris et al. 1984; Salowe et al., 1987). The R_f's for phosphate and pyrophosphate were 0.74 and 0.47, respectively. The strips containing phosphate and pyrophosphate were cut out and extracted with 1 mL of 1 M TEAB. A portion of the extracted solution was counted. A total of 1800 cpm (92%) was detected in the phosphate spot. In the control experiment, quantitative radioactivity was also recovered as phosphate.

Preparation of Mutant C439R1s. The procedures are identical to those described in the preceding papers (Mao et al., 1992a,b). The following primers were used to make the corresponding mutant R1s: C439Y, TCTAACCTGTATCTGGAGATA; C439H, TCTAACCTGCACCTGGAGATA; C439W, TCTAACCTGTGGCTGGAGATA. The mutant genes were sequenced only in the region of the mutation. The mutant proteins were isolated as previously described.

RESULTS

Characterization of Mutant C439SR1. In the absence of X-ray crystallographic information, if a residue is thought to play an essential role in catalysis, the structural integrity of the mutant must be established. As indicated below, affinity chromatography, substrate binding studies, steady-state kinetic analysis, and CD spectroscopic analysis all confirm that C439SR1 is structurally similar to the wt-R1.

Purification by dATP Affinity Chromatography. The mutant and nonmutant RDPRs were purified from *E. coli* lysates using a *p*-aminophenyl-dATP–Sepharose affinity column that is proposed to function by recognition of the dATP allosteric binding site of R1 (Berglund & Eckstein, 1974). The amount of mutant protein recovered is typically 1.0–1.5 mg/g of cells, similar to that observed with wt-R1 and all the mutants prepared thus far, and suggests that allosteric binding site remains intact in C439SR1.

Ultrafiltration Studies To Investigate GDP Binding. In addition to the integrity of the allosteric binding site, it is

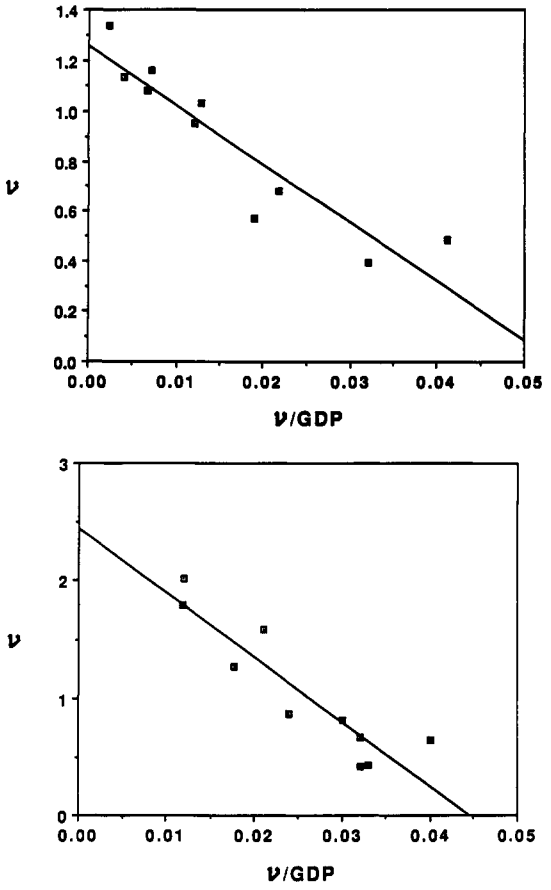

FIGURE 1: (A, top) Scatchard plot of GDP binding to C439SR1 in the presence of allosteric effector TTP. (B, bottom) Scatchard plot of GDP binding to wt-R1 in the presence of allosteric effector TTP.

important to establish the integrity of the substrate binding site. Previous equilibrium dialysis studies of von Döbeln and Reichard (1976) have concluded that the NDP substrates binding to the R1 subunit. For GDP in the presence of an allosteric effector analog dTMP-PCH_2P,[1] they found 1.3 binding sites/R1 with a K_d = 22 μM at 20 °C. Soderman and Reichard (1986) developed a nitrocellulose filter binding assay and Ormö and Sjöberg (1990) developed an ultrafiltration method that have allowed measurement of K_d's for the allosteric effectors. Ormö and Sjöberg reported a K_d of 24 μM at 25 °C with two binding sites for the substrate GDP with wt-R1 in the presence of the allosteric effector TTP. This method has been utilized to determine the K_d for GDP with C439SR1 as well as for the wt-R1. The results are shown in Figure 1A and B. A K_d = 28 μM and 1.25 binding sites was observed with C439SR1. Analogous studies with the wt-R1 indicate a K_d = 53 μM and 2.5 binding sites. The K_d values are similar for mutant and wild-type R1. While the number of binding sites appears to be different, these differences may not be significant given the scatter in the data and the inherent limitations in the methods due to the high K_d values.

Steady-State Kinetic Studies with C439SR1. Recently we investigated the steady-state kinetic mechanism of RDPR. The K_m value describing the interaction R1 with R2 at saturating CDP and allosteric effector ATP was determined to be 0.16 μM. This value is similar to the value recently obtained by Climent et al. (1991). The interaction of C439SR1 with R2 was investigated by examining the ability

[1] The triphosphate analog in which a methylene bridge is placed between the β- and γ-phosphates of TTP.

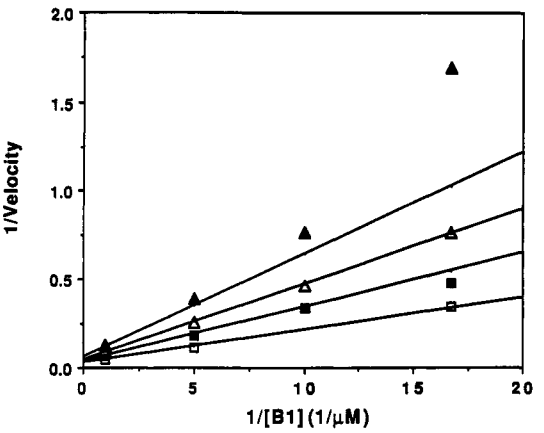

FIGURE 2: C439SR1 inhibition of wt-R1's interaction with R2. The [C439SR1] is (□) 0, (■) 1.5, (△) 3, and (▲) 5 μM.

of this mutant to act as a competitive inhibitor for R1. The results of this kinetic experiment are shown in Figure 2. Analysis of the data by the methods of Cleland indicates equally good fits to the equations describing competitive and noncompetitive inhibition. At the micromolar concentrations of C439SR1 used in the present assays, a control was routinely run in the absence of wt-R1 to establish the amounts of dCDP produced by the contaminating wt-R1 in the mutant. This complication may account for the scatter in the data (Figure 2). The competitive fit gives a K_{is} = 1.40 μM and a K_a = 0.58 μM, while the noncompetitive fit gives a K_{is} = 2.38 μM, K_{ii} = 4.3 μM, and a K_a = 0.66 μM. In addition, a deviation from linearity may result if a ping-pong mechanism for nucleotide reduction is observed (Thelander, 1974). The TR and R2 could both be competing for binding to R1. Despite these problems, these results support the notion that the ability of R1 to bind to R2 has not been substantially altered by conversion of C439 to a serine.

Structural Characterization of C439SR1 Using CD. A method routinely used to examine the structure of mutant proteins is circular dichroism (CD). The CD spectrum of C439SR1 appears to be identical to that of the wt-R1. By use of the variety of methods described above, the structure of the C439SR1 appears to be similar to although not identical to wt-R1.

Function of C439. If the function of C439 is to initiate abstraction of the 3′-hydrogen of the substrate, then mutation of this C to a S should result in a protein with *no* catalytic activity. As discussed in the preceding papers, however, given that the *nrd*A gene product R1 is essential for the viability of the *E. coli*, there are no *E. coli* that lack this gene. Hence all of the mutants of R1 that have thus far been prepared are contaminated with chromosomally encoded wt-R1. The level of wild-type contamination appears to be typically in the range of 1.5–3%, based on activity measurements. Assays of C439SR1 activity with CDP as substrate (ATP as effector) and either thioredoxin/thioredoxin reductase/NADPH or dithiothreitol as reductants are summarized in Table I. The only product of the reaction is dCDP, identified by HPLC analysis of the nucleoside. The rate of its production is within the range of that expected for contaminating wt-R1. To show that this residual activity is due to contaminating wt-R1, a method was sought to remove the wild-type contaminant without having any effect on the C439SR1. Previous studies of Salowe et al. (1987) have shown that RDPR is stoichiometrically inactivated by the mechanism-based inhibitor N_3-UDP. In fact, incubation of this inhibitor with enzyme results in destruction of the tyrosyl radical on R2, cleavage of the 3′

Table I: Activity of C439SR1

	reductants		
	TR/TRR V_{max} (nmol min^{-1} mg^{-1})	K_m CDP (μM)	DTT[c] V_{max} (nmol min^{-1} mg^{-1})
wt-R1			
R1/R2 (1:1)[a]	660		
R1/R2 (1:5)[b]	1300	73	42
C439SR1			
R1/R2 (1:1)[a]	44.5 (3.4%)	160	1.5 (3.5%)

[a] A 1:1 molar ratio of R1 to R2 was used. Given that the K_m of R1 for R2 is 0.16 μM under the conditions of a typical assay, R1 is not saturated with R2. With the mutant proteins, which require much higher concentrations of R1 to R2 to assay product formation, changing the ratio of R1 to R2 does not appear to alter the activity. [b] A 1:5 molar ratio of R1 to R2 gives maximal activity. [c] Reactions were carried out with 10 mM DTT.

C–H bond of N_3UDP, and stoichiometric production of PP_i, uracil, and N_2. In addition, the R1 subunit is stoichiometrically alkylated by the 2-methylene-3(2*H*)-furanone [Salowe et al. (1987); unpublished results]. We reasoned that this inhibitor would rapidly and efficiently inactivate the contaminating wt-R1 and that this inactivation would be accompanied by ~0.015–0.030 equiv of PP_i based on the estimated amounts of wt-R1 in the mutant preparations. On the other hand, if C439SR1 was slowly inactivated by N_3UDP, one would expect stoichiometric amounts, ~1.0 equiv of PP_i to accompany this inactivation. If C439S of R1 is in fact the X• in our proposed mechanism [Scheme I, Mao et al. (1992b)], then the C439SR1 mutant should be unable to catalyze the cleavage of the 3′ carbon–hydrogen bond of the substrate and no PP_i should be produced. C439SR1 was therefore inactivated with N_3UDP under several different sets of conditions. In the first case, the experiment was designed to set a lower limit of detection of dCDP production. In the second set of experiments, the amount of PP_i accompanying inactivation was quantitiated in an attempt to establish that PP_i production is the result only of the presence of contaminating wt-R1.

To determine the rate of dCDP production, C439SR1 was inactivated by incubation with 1 mM N_3UDP for 30 min. The N_3UDP was removed by the procedure of Penefsky (1977), and the activity of C439SR1 was determined by incubation with [U-^{14}C]CDP of very high specific activity (7.6×10^6 cpm/μmol). The rate of dCDP production was less than 0.45 nmol min^{-1} mg^{-1}, less than $^1/_{2888}$ the rate of turnover of the wt-R1 (0.03%) and the lower limit of detection using this assay method. Thus, C439SR1 appears to be inactive.

The second set of experiments was designed to detect the amount of PP_i accompanying C439SR1 inactivation. This experiment was anticipated to be technically difficult to carry out due to the low levels of PP_i expected to accompany inactivation and the small amounts of contaminating P_i and PP_i always present in preparations of [β-^{32}P]N_3UDP of very high specific activity (3.5×10^8 cpm/μmol). The inactivation of C439SR1 with N_3UDP (50 μM) was carried out in the presence of F^-, an inhibitor of low levels of contaminating phosphatases that may be present in the protein preparation. In addition, a control experiment was carried out in which R2 was replaced with met-R2 (the tyrosyl radical reduced and hence inactivated R2). Subsequent to inactivation, carrier PP_i (10 μmol) was added to the reaction mixture, which was then extracted twice with acid-washed charcoal to remove the unreacted [β-^{32}P]N_3UDP. The remaining radioactivity was then chromatographed on a DEAE–Sephadex A-25 anion exchange column. The elution profile of P_i and PP_i from the

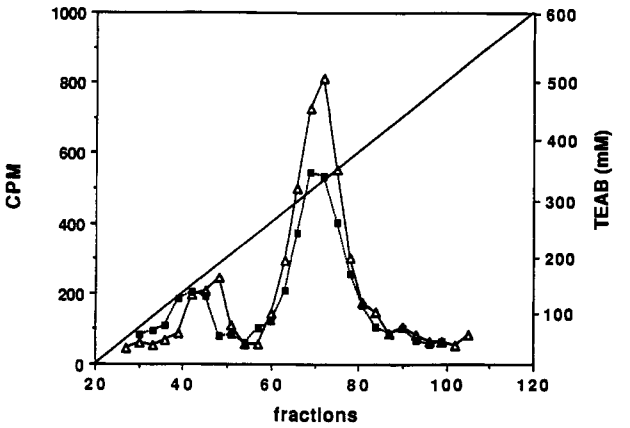

FIGURE 3: Elution profile of products produced on interaction of [β-^{32}P]N_3UDP with C439SR1 and R2: experiment (△); control, in which met R2 replaced R2 (■).

Table II. Quantitation of Pyrophosphate Release upon Incubation of C439SR1 with N_3UDP

trial	control[a]	experiment	difference
1	0.037	0.053	0.016
2	0.038	0.046	0.010
3	0.089	0.112	0.023

[a] Control uses met R2 (Y• reduced and hence inactive R2) in place of R2.

experiment and the control are shown in Figure 3. The results of three separate experiments are reported in Table II. The amount of PP_i reported is determined by subtracting the PP_i observed in the control from that observed in the experiment. An average value of 0.016 equiv of PP_i/equiv of C439SR1 is observed, consistent with the notion that inactivation of the contaminating wt-R1 is responsible for PP_i production and loss of ability to produce dCDP.

The radioactivity eluted in the putative "PP_i" pool was further characterized to establish its identity. The material was rechromatographed on a polyethyleneimine (PEI) thin-layer plate before and after treatment with inorganic pyrophosphatase. This system separates N_3UDP from PP_i and P_i (R_f = origin, 0.47, and 0.74, respectively). In the case of the inorganic pyrophosphatase treated material, all of the radioactivity migrated with P_i.

The observation that only 1–2 mol % PP_i/mol of C439SR1 is observed and that the activity of C439SR1 is now 0.03% that of the wt-R1 allows us to conclude that C439 is essential for catalysis.

Thioredoxin-like Activity Attributed to C439SR1. A second way to avoid the problem associated with the presence of contaminating wt-R1 activity in the mutant preparations would involve a single-turnover experiment. If the prereduced C439SR1 in the absence of any external reductant were incubated with [^{14}C]CDP, then for every mole of R1 present only 0.03–0.06 mol of dCDP (two putative active sites per R1) would be produced (the amount due to the 1.5–3% contaminating wild-type activity).

The results of a typical one-turnover experiment are shown in Figure 4. In the limit, after 200-min incubation, two dCDPs are produced, although the rate of this reaction is extremely slow. If the function of C439 is to remove a hydrogen atom from the substrate, then the mutant protein should be unable to catalyze dCDP production. The observation of two dCDPs therefore seems inconsistent with our proposed role for this residue. However, suppose that the redox-active thiols of C439SR1, either C754, C759, or C225 and C462 are capable

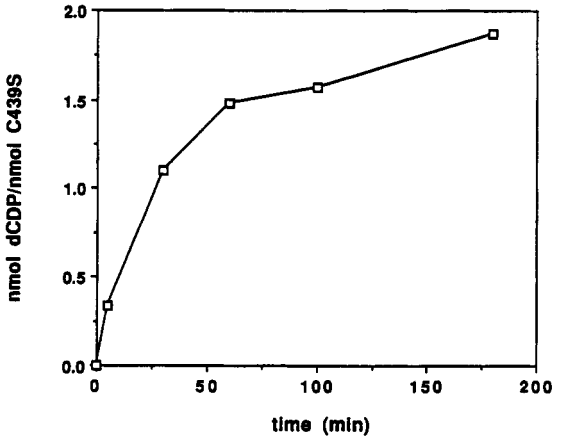

FIGURE 4: Typical one-turnover experiment. Prereduced C439SR1 is incubated with R2 and [^{14}C]CDP and analyzed for dCDP production in the absence of any external reductants.

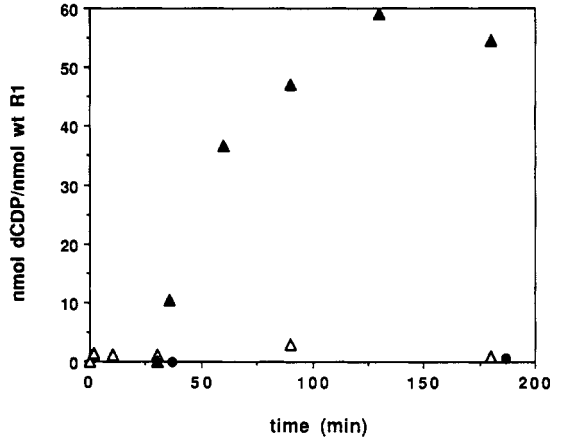

FIGURE 5: Catalysis of wt-R1-mediated reduction of CDP to dCDP by N_3UDP-inactivated C439SR1. C439SR1 possesses thioredoxin-like activity. (△) incubation of wt-R1, R2 in the absence of external reductant; (▲) addition of 30 equiv of N_3UDP-inactivated C439SR1; (●) control incubation of R2 followed by addition of 30 equiv of N_3UDP-inactivated C439SR1.

of acting as a reductant for the contaminating wt-R1 in much the same way that thioredoxin reduces wt-R1. One would then expect that the amounts of dCDP produced would be dictated by the amount of mutant protein present. Specifically for each mole of C439SR1 present, 2 mol of dCDP could be produced. C439SR1 would thus have thioredoxin-like activity. To test this model, prereduced wt-R1 was incubated with [^{14}C]CDP in the absence of any external reductant and approximately 2 equiv of dCDP and oxidized wt-R1 were observed. As expected, dCDP production was complete at the first time point (1 min) (Figure 5). After 20 min, C439SR1 (a 30-equiv excess with respect to oxidized wt-R1) that had been inactivated with N_3UDP was then added to the reaction mixture. dCDP production was again observed, and in the limit 2 mol of dCDP/mol of C439SR1 was observed (Figure 5). A control experiment run under identical conditions except that wt-R1 was omitted from the initial incubation revealed after 150 min that no dCDP was produced. The rate of dCDP production was also proportional to the amount of wt-R1 added (data not shown). These studies make the intriguing observation that in spite of the fact that C439SR1 cannot reduce nucleotides, it has the ability to function as a reductant of the wt-R1, albeit at greatly reduced rates in comparison with thioredoxin.

Characterization of Additional C439 Mutants. C439HR1 and C439WR1 and double mutant C439Y–C759SR1 were prepared in an effort to test the hypothesis that C439 is converted to a thiyl radical required to initiate catalysis. Observation by spectrophotometric methods of oxidized H, W, or Y of any one of these respective mutants would support this model. Experiments monitoring tyrosyl radical loss were therefore initiated in an attempt to test this hypothesis. None of these mutants caused significant tyrosyl radical loss of R2 in the presence of the allosteric effector TTP and substrate CDP. A loss of 0.02 equiv/60 min was observed in all three cases, similar to a control with wt-R2. Since no tyrosyl radical loss is observed, each of these mutants was asayed for their ability to reduce dCDP. The rate of dCDP production varied from 6 to 20 nmol min^{-1} mg^{-1} within the range of the contaminating wt-R1 activity. These mutants, as with C439SR1, appear to be inactive.

DISCUSSION

Site-directed mutagenesis studies have allowed us to propose a model for nucleotide reduction in which five cysteines are required. Two cysteines, 754 and 759, are postulated to be involved in the transfer of electrons between thioredoxin and the active site cysteines of R1 (Lin et al., 1987; Mao et al., 1992a,b), two cysteines, 225 and 462, are postulated to be directly involved in substrate reduction, and the fifth cysteine, 439, is proposed to be directly involved in hydrogen atom abstraction from the C-3′ position of the substrate.

Since C439SR1 is proposed to be essential for catalysis and hence would possess no catalytic capabilities, it is very important to establish that it is structurally intact. This is not an easy task in the absence of X-ray crystallographic information. However, a number of methods have been investigated to establish the mutant's ability to bind R2, substrate, the allosteric effectors.

The ability to isolate mutant protein using an affinity column generated from a derivative of an allosteric effector as well as the amount of protein isolated by this procedure indicates that the protein is produced and its effector sites are intact. In addition, the CD spectrum of wt-R1 and C439SR1 are identical, indicating no gross structural changes. To test whether the substrate binds to the mutant protein, we were limited in choice of methodology due to high K_d values previously reported for substrates (von Dobeln & Reichard, 1976). GDP was selected as the substrate of choice as previous equilibrium dialysis studies of von Dobeln and Reichard indicated a K_d = 22 μM using a TTP derivative in which the oxygen between the β- and γ-phosphates of the triphosphate moiety was replaced by a methylene group. More recent studies of Ormö and Sjoberg (1991) using an ultrafiltration binding assay also gave a $K_d \approx 26$ μM. We used this method to measure the affinity of wt-R1 and C439SR1 for GDP. K_d values of 55 and 23 μM, respectively, were measured, similar to those previously reported. However, the Scatchard analysis revealed a value of 2.5 GDPs/wt-R1, in comparison with the value of 2.0 previously reported by Sjöberg et al. and 1.3 previously reported by von Döbeln and Reichard. The interaction of C439SR1 with GDP revealed a value of 1.25. Thus substrate appears to bind to both wild type and mutant. It should be noted, however, that the high K_d observed with GDP results in substantial scatter in the data (Figure 1A and B) and represents close to an upper limit of a K_d determinable by this method. The scatter in the data suggests that differences in the number of binding sites observed with the wt-R1 and C439SR1 may not be statistically significant.

To assess the ability of C439SR1 to interact with R2, it was examined as a competitive inhibitor of R1 in a steady-state

kinetic assay. The data shown in Figure 2 give a slightly better fit to the equation for noncompetitive inhibition than to those for competitive inhibition. There is however substantial scatter in the data due to several problems. First, corrections are required for the contaminating wt-R1 in the C439SR1 mutant. Second, the kinetic mechanism of this reaction, while suggested to be ping pong (Thelander, 1974), has not yet been adequately defined. If a ping-pong mechanism is operative, then both TR and R2 will be competing for binding to R1, potentially giving rise to the nonlinearity observed. Efforts in our hands to define the kinetic mechanism, however, have thus far failed to allow a distinction to be made between a sequential and ping-pong mechanism. With fits to either competitive or noncompetitive inhibition the K_i's are 1–2 μM, 5–10 times greater than the K_m of 0.16 μM of R1 for R2. These data indicate that C439SR1 prevents interaction of R1 with R2 at micromolar concentrations. Thus, a variety of methods suggest C439SR1 is intact, but that it appears to be subtly different from wt-R1 in ways that will ultimately be distinguishable only by crystallographic methods.

The major problem with defining the role of the R1 mutants has been a technical one, in that *E. coli* requires the *nrdA* gene product (the R1 subunit) for survival. All of the mutants prepared thus far contain small amounts of contaminating wt-R1. Preliminary studies of Mao et al. (1992a,b) indicate that an N-terminal epitope-tagged R1 can be partially resolved from wt-R1 by anion exchange chromatography due to the additional charges present in the epitope. This paper describes two additional approaches to alleviate this problem, which have allowed further definition of the catalytic capabilities of C439SR1.

The first solution involves taking advantage of the fact that wt-RDPR is stoichiometrically inactivated by incubation with N_3UDP. This inactivation is accompanied by destruction of the tyrosyl radical, cleavage of the 3′ carbon–hydrogen bond of the substrate, formation of uracil, PP_i, N_2, and a nitrogen-centered radical, and alkylation of R1 by the 2-methylene-3(2*H*)-furanone inactivating the R1 subunit [Salowe et al. (1987) and unpublished results]. If C439SR1 is really catalytically incompetent, then its interaction with N_3UDP should result in no chemistry and no PP_i should be produced. If C439SR1 is catalytically competent, then after long time periods 1 equiv of PP_i should be produced. Thus we reasoned that the wt-R1 contaminant (probably present as a heterodimer) of C439SR1 could be destroyed by incubation with [^{32}P]N_3UDP and that the amount of this wt-R1 present could be assessed by the release of $^{32}PP_i$. Incubation of C439SR1 and contaminating wt-R1 with N_3UDP followed by removal of the inhibitor and assay for activity revealed that the enzyme possessed less than $^1/_{2888}$ (0.03%) the activity of the wild-type protein. This value is very close to the lower limit of detection of our assay given the background problems.[2]

In a second experiment, the amount of $^{32}PP_i$ accompanying this inactivation was quantitated. As indicated in Table II, both P_i and PP_i were found in the experiment and the control, the latter involving met-R2 in place of R2. In all experiments in which analogs of [^{32}P]NDPs of high specific activity have been investigated, low levels of these contaminating species exist. The experiment and control were each repeated three times, and each time the experiment contained larger amounts of PP_i than the control. The difference between the experiment and the control indicates that the PP_i produced is on the average 1.6% of the amount of C439SR1. The low activity of C439SR1 mutant and the fact that the activity is knocked out by N_3-UDP resulting in PP_i production at the level expected for the contaminating wt-R1 suggests that C439 is essential for catalysis.

The second solution to the contamination problem might involve the execution of one-turnover experiments. Each reduction of an NDP to a dNDP is accompanied by concomitant oxidation of two thiols to a disulfide within each promoter of R1. In the absence of any external reductants, the enzyme is inactive after a single turnover (Thelander, 1974; Lin et al., 1987). Thus if dCDP production is only associated with the amounts of contaminating wt-R1 present in the mutant protein, then under one turnover conditions only 0.03–0.06 equival (two promoters and hence two active sites per R1) would be produced. As indicated in Figure 4, however, two dCDPs were produced, although the time course for their production was very slow. At first glance, this result seems to imply that C439SR1 is in fact catalytically active. However, an alternative interpretation must be considered. C439SR1 still has four redox active thiols present per protomer (225, 462, 754, 759). Suppose that the C754 and C759 could function, albeit at greatly reduced efficiency, as a reductant for wt-R1 in much the same way thioredoxin functions in this role. One would then expect that eventually 2 dCDPs/C439SR1 would be produced. This is in fact the experimental result (Figure 4).

To confirm this hypothesis, therefore, C439SR1 was inactivated with N_3UDP. The inactivated mutant protein was then added to oxidized wt-R1. The mutant protein successfully served as a reductant allowing multiple turnovers of wt-R1 (Figure 5). In the absence of oxidized wt-R1, under otherwise identical conditions, no dCDP was observed. A second control, adding a 30-equiv excess of the double mutant C754S and C759SR1 in place of N_3UDP-inactivated C439SR1, revealed no turnover over that observed in the control. Thus, C439SR1 appears to possess low levels of a thioredoxin-like activity presumably by using its C-terminal cysteines C754 and C759. While this activity is novel and unanticipated, it prevents the use of one-turnover experiments to define the essentiality of C439.

As a further test of the model that the Y122• on R2 generates a C439 radical on R1 via long-range electron transfer, a variety of site-directed mutants of residue 439 were prepared in which cysteine was replaced with amino acids with the capacity to be oxidized (H, W, Y). All three of these residues have been implicated in radical-dependent processes in other systems (Li et al., 1991; Babcock et al., 1990; Stubbe, 1988; Ono & Inoue, 1991). If any of these residues were oxidized to a radical via reduction of Y122, they might be detectable using UV/visible spectroscopy. Each of these mutants was analyzed for catalytic activity, for loss of tyrosyl radical, and for formation of alternative radicals. Unfortunately none of the mutants had any detectable activity above that observed with the contaminating wt-R1. Furthermore, incubation of these mutants with CDP, TTP, and R2 resulted in no loss of tyrosyl radical during a 60-min incubation above that observed with a control. The nonresponsiveness of these mutants can be rationalized if the long-range electron transfer between R1 and R2 requires a very precise alignment apparent only if both the substrate and its appropriate alllosteric effector are bound. It is likely that differences in steric bulk and electronic properties of H, Y, and W in comparison with C prevent this required alignment. The oxidation potentials of these residues in the protein environment of R1 may also be inappropriate

[2] Commercially available [U-^{14}C]CDP possesses a small amount of material that comigrates with cytosine. The amount of this material increases with storage.

to undergo oxidation mediated by a tyrosyl radical. Efforts to replace C439 with an isosteric amino acid such as selenocysteine, which is more easily oxidized than cysteine, are in progress.

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Registry No. RDPR, 9047-64-7; Cys, 52-90-4; GDP, 146-91-8; CDP, 63-38-7.