# Kinetics in the Pre-Steady State of the Formation of Cystines in Ribonucleoside Diphosphate Reductase: Evidence for an Asymmetric Complex[†]

Hans K. Erickson*

*Department of Chemistry, 0506, University of California at San Diego, 9500 Gilman Drive, La Jolla, California 92093-0506*

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ABSTRACT: Two folded polypeptides, designated R1 and R2, respectively, combine in an as yet undefined stoichiometry to form ribonucleoside diphosphate reductase (ribonucleotide reductase) from *Escherichia coli*. Two pairs of cysteines in each R1 protomer have been implicated in the enzymatic mechanism. One pair, cysteines 225 and 462, is located in the active site of the enzyme and forms a cystine concomitant with the reduction of the ribonucleotide. The other pair, cysteines 754 and 759, is located near the carboxy terminus and is thought to reduce the cystine in the active site by disulfide interchange; either thioredoxin or glutaredoxin is then thought to reduce the cystine that results. Rapid quenching and site-directed immunochemistry have been used to follow the formation of the cystine in the active site and the peripheral cystine simultaneously during the pre-steady state. Prereduced R1 dimer of ribonucleoside diphosphate reductase, in the presence of ATP and CDP, was mixed with R2 dimer in an apparatus for quench flow. The reaction was quenched with a solution of acetic acid and *N*-ethylmaleimide, the protein was then precipitated with trichloroacetic acid, and the precipitate was separated into two portions. The percent of the cystine in the active site in one of the portions was determined as described previously [Erickson, H. K. (2000) *Biochemistry 39*, 9241−9250]. A similar method was employed to determine the percent of the peripheral cystine in the other portion of the precipitate. It was found that while the formation of both of these cystines was initiated by the addition of R2 dimer, presumably as products of the reduction of CDP, the peripheral cystine appeared to form more rapidly and in a higher yield than the cystine in the active site. These results demonstrate that the formation of the cystine between cysteines 754 and 759 of ribonucleotide reductase from *E. coli* is kinetically competent. A mechanism consistent with the prior formation of the cystine between cysteine 225 and cystene 462 as well as the kinetics for the formation of each cystine with time is presented. Because twice as much of the peripheral cystine than cystine in the active site had formed during the pre-steady state, it follows that the enzymatically competent complex between R1 dimers and R2 dimers cannot be symmetric.

Ribonucleoside diphosphate reductase (ribonucleotide reductase)[1] catalyzes the 2′-reduction of ribonucleoside diphosphates to produce the deoxyribonucleoside diphosphates necessary for biosynthesis and repair of DNA. Ribonucleotide reductases have been divided into three classes based on their requirements for cofactors. Ribonucleotide reductase from *Escherichia coli* belongs to class 1 that includes the mammalian and herpes viral enzymes.

Two folded polypeptides, designated R1 and R2, respectively, combine to form the active enzyme from *E. coli*. Crystallographic molecular models are available for the native R1 dimer (*1*, *2*) and the native R2 dimer (*3*) but not for any complex of the two. It has been proposed that an equimolar amount of R1 dimer and R2 dimer combine to produce a rotationally symmetric holoenzyme (*3*, *4*), but the stoichiometry and symmetry of the active complex has not yet been defined. The R1 protomer contains the site at which all four ribonucleotides are reduced, and the R2 protomer contains a binuclear cluster of iron that defines the enzymes from class 1. This binuclear cluster of iron generates and maintains a tyrosyl radical located at tyrosine 122 of the R2 protomer (*5*).

R1 protomers from ribonucleotide reductases of class 1 contain five conserved cysteines. These cysteines are located at positions 225, 439, 462, 754, and 759 in the R1 polypeptide from *E. coli*. Each is a participant in the various enzymatic mechanisms that have been proposed (*2*, *6*−*8*). The cysteines at 225, 439, and 462 are located within the active site of the crystallographic molecular model of the R1 dimer (*2*). Cysteine 439 is believed to form a thiyl radical that initiates catalysis by removing the hydrogen from the 3′ carbon of the ribonucleotide (*9*). Cysteine 225 and cysteine 462 supply the hydrogen and the electron required for the reduction of the 2′ carbon of the ribonucleotide (*10*, *11*). At the completion of one turnover, they have become a cystine (*12*) that must be reduced for the enzyme to complete additional turnovers. Reducing equivalents for ribonucleotide reductases from class 1 and class 2 are supplied by either reduced thioredoxin or reduced glutaredoxin. Thioredoxin

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* Present address: Department of Chemistry, University of Utah, 315 S. 1400 E. Room 2020, Salt Lake City, UT 84112. Phone: 801-581-7493. Fax: 801-581-4391. E-mail: erickson@chemistry.chem.utah.edu.

[1] Abbreviations: ribonucleotide reductase, ribonucleoside-diphosphate reductase; HEPES, *N*-(2-hydroxyethyl)-*N*′-(2-sulfoethyl)piperazine; AQHC, 6-aminoquinolyl-*N*-hydroxysuccimidyl carbamate; NEM, *N*-ethylmaleimide; FMOC, 9-fluorenylmethyloxycarbonyl; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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and glutaredoxin, however, are incapable of reducing the cystine in the active site directly (*10*, *13*, *14*). Mutation of cysteine 754 or cysteine 759 to alanine or serine inactivates the enzyme (*10*), but activity is restored by the addition of the external reductant dithiothreitol. When either of these two mutants are prereduced and mixed with CDP in the absence of any reductant, only half as much deoxycytidine diphosphate is produced as with the wild-type enzyme (*10*) under the same circumstances. Taken together, these results suggest that cysteine 754 and cysteine 759 transfer reducing equivalents from thioredoxin or glutaredoxin to the active site by reducing the cystine in the active site and then oxidizing thioredoxin or glutaredoxin by disulfide interchange, but this has never been observed directly. There is no electron density in the maps that can be assigned to the segment of the protein containing cysteine 754 and cysteine 759 (*1*, *2*).

To investigate the role of these cysteines in the enzymatic mechanism of ribonucleotide reductase, experiments were performed to determine simultaneously the rates for the formation of the cystine between cysteine 225 and cysteine 462 and the cystine between cysteine 754 and cysteine 759 in the pre-steady state.

## EXPERIMENTAL PROCEDURES

*Materials.* The R1 dimer ($\epsilon_{272}$ = 90 $mM^{-1}$ $cm^{-1}$) of *E. coli* ribonucleotide reductase with a specific activity of 1.3−1.6 $\mu$mol of oxidized thioredoxin $min^{-1}$ (mg R1 dimer)$^{-1}$ at the optimal concentration of R2 dimer was isolated from the overproducing strain K38pMJ1 (*15*). The R2 dimer ($\epsilon_{277}$ = 150 $mM^{-1}$ $cm^{-1}$) of *E. coli* ribonucleotide reductase with a specific activity of 6 $\mu$mol oxidized thioredoxin $min^{-1}$ (mg R2 dimer)$^{-1}$ at the optimal concentration of R1 dimer was purified from the overproducing strain C600pMB1 (*16*). These strains of *E. coli*, each overproducing one of the two component proteins of ribonucleotide reductase, and protocols for the purification of the proteins were generously supplied by Dr. JoAnne Stubbe of the Massachusetts Institute of Technology. Thioredoxin from *E. coli* with a specific activity of 38 units $mg^{-1}$ was isolated from overproducing strain SK6517 (*17*), and thioredoxin reductase with a specific activity of 240 units $mg^{-1}$ was isolated from strain A326/pTrR301 (*18*, *19*). A unit for the latter two enzymes is defined as a change of one unit of absorbance at 412 nm [the wavelength of maximum absorbance for the 2-nitro-5-thiobenzoate formed from 5,5′dithiobis(2-nitrobenenzoic acid)] $min^{-1}$ in the colorimetric assay (*20*). $N^{\alpha}$-Fluorenylmethyloxycarbonyl ($N^{\alpha}$-FMOC)[1] amino acids, 1-hydroxybenzotriazole hydrate, and *p*-alkoxybenzyl solid phase were purchased from either Bachem or Calbiochem-Novabiochem. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] bovine serum albumin, Sephadex G-25, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide (NEM)[1], dithiothreitol, nucleotides, and NADPH were purchased from Sigma Chemical; glutamyl endopeptidase from *Staphylococcus aureus* strain V8 and chymotrypsin, from Worthington; 1,3-diisopropylcarbodiimide, *N*-methylpyrrolidinone, ethanedithiol, hydrindantin, piperidine, and trichloroacetic acid from Aldrich Chemical; trifluoroacetic from Halocarbon Products; ninhydrin and dimethylpimilimidate, from Pierce Chemical; protein A Affigel, from Bio-Rad Laboratories; thermolysin, from Calbiochem-Novabiochem.

*Assay of Enzymatic Activity.* The coupled enzyme assay of Thelander et al. (*21*) was slightly modified (*12*) and used to determine the steady-state activity of R1 dimer and R2 dimer.

*Synthesis of PSIQDDGCESGACK*. The peptide PSIQDDGCESGACK was synthesized on the solid phase using the fluorenylmethyloxycarbonyl strategy (*22*). $N^{\alpha}$- Fluorenylmethyloxycarbonyl-$N^{\epsilon}$-(*tert*-butyloxycarbonyl)-L-lysine was attached to a *p*-alkoxybenzyl solid phase with diisopropylcarbodiimide. The preformed hydroxybenzotriazole esters of $N^{\alpha}$-FMOC-*S*-(triphenylmethyl)-L-cysteine, $N^{\alpha}$-FMOC-L-alanine, $N^{\alpha}$-FMOC-L-glycine, $N^{\alpha}$-FMOC-$O^{\beta}$-butyl-L-serine, $N^{\alpha}$-FMOC-L-glutamic-$O^{\gamma}$-butyl ester, $N^{\alpha}$-FMOC-L-aspartic-$O^{\beta}$-butyl ester, $N^{\alpha}$-FMOC-L-glutamine, $N^{\alpha}$-FMOC-L-isoleucine, and $N^{\alpha}$-FMOC-L-proline were then added sequentially to complete each cycle of the synthesis. The product, PSIQDDGCESGACK was cleaved from the solid phase, and all protecting groups were removed by treatment with 95:2:2:1 trifluoroacetic acid, ethanedithiol, thioanisole, and water for 10 h. Trifluoracetic acid was removed under reduced pressure, and the remaining residue was dissolved in 10% acetic acid and extracted with diethyl ether. The aqueous phase was lyophilized, and the crude peptide was purified by reverse phase high-pressure liquid chromatography (HPLC).[1]

The synthetic peptide was hydrolyzed in 6 M HCl for 45 min at 160 °C, and the amino acid composition was determined. Total enzymatic digestion followed by amino acid analysis was also used to ensure that the removal of all protecting groups was complete. The composition of amino acids after each of these treatments was in agreement with the expected values for the synthetic peptide.

*Preparation of IQDDGCE.* The synthetic peptide PSIQDDGCESGACK was digested with thermolysin and the glutamyl endopeptidase from *S. aureus* strain V8 to remove the amino-terminal dipeptide and the carboxy-terminal pentapeptide, respectively. The product IQDDGCE was purified by HPLC, and its identity was confirmed by amino acid analysis.

*Preparation of Immunoadsorbents*. A haptenic conjugate of the peptide IQDDGCE and bovine serum albumin was prepared by the method described by Ewalt (*23*). Rabbit antipeptide antisera were elicited by an initial subcutaneous injection of a 1:1 suspension of the haptenic conjugate (300 $\mu$g) and Freund's complete adjuvant followed by subsequent booster injections with incomplete Freund's adjuvant and complete Freund's adjuvant after two weeks and one month, respectively. The synthetic peptide IQDDGCE was used to prepare an affinity column (*23*) to isolate immunoglobulins capable of recognizing the amino-terminal sequence IQDDG-. The affinity purified immunoglobulins were in turn covalently attached to protein A agarose with dimethylpimilimidate (*23*) to produce the immunoadsorbent. The immunoadsorbent had the capacity to bind 8 nmol of the synthetic peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]E. Preparation of the immunoadsorbent used to retrieve peptides containing cysteine 225 and cysteine 462 has been described previously (*12*).

*Quench Flow Measurements*. Measurements by quench flow were performed using a KinTek RGF-3 instrument for quench flow as described previously (*12*) with slight modifications. Prereduced R1 dimer was treated with hydroxyurea as described by Stubbe[2] to destroy any contami-

nating R2 dimer, mixed with ATP and CDP in HEPES buffer containing EDTA and $MgSO_4$, and loaded into one syringe of the quench flow apparatus. The allosteric effector, ATP, increases the rate of reduction of CDP. To minimize oxidation of the peripheral cysteines, the pH of the 5 mM HEPES buffer in this syringe was 6.5. The other syringe contained R2 protein in 100 mM HEPES buffer containing EDTA and $MgSO_4$. Upon the mixing of the effluents from the two syringes, the final concentrations were 15 $\mu$M R1 dimer, 30 $\mu$M R2 dimer, 1.6 mM ATP, 1 mM CDP, 1 mM EDTA, and 15 mM $MgSO_4$. The HEPES buffer in the syringe containing R2 protein was prepared so that upon mixing the final buffer would be 52 mM HEPES, pH 7.6. The mixture was quenched with at least 30% acetic acid and 8 mM NEM and after removing the sample from the apparatus the protein was precipitated by adding four volumes of 8% trichloroacetic acid.

*Enzymatic Digestions and Immunoadsorption*. Peptides containing cysteine 225 or the cystine between cysteine 225 and cysteine 462 were isolated from precipitated ribonucleotide reductase as described previously (*12*). A similar strategy was employed to isolate carboxy-terminal peptides containing cysteine 754 and cysteine 759. Protein that had been precipitated with trichloroacetic acid was digested with thermolysin (1 mg $mL^{-1}$) in 4 mM NEM and 50 mM ammonium bicarbonate, pH 7.8, for 2 h at 37 °C. Thermolysin was inhibited with 30 mM EDTA, and the digest was passed over the immunoadsorbent specific for peptides containing the amino-terminal sequence IQDDG-. The immunoadsorbent was washed with 40 mL of with 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4, and specifically adsorbed peptides were eluted with 0.5 M acetic acid. Before another sample was applied, the immunoadsorbent was successively washed with 15 mL of 0.1 M sodium phosphate, pH 2.5; 15 mL 0.5 M acetic acid; 15 mL 0.1 M phosphate, pH 2.5; and 15 mL of 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4.

*Fluorescent Modification of Affinity Purified Peptides*. Modification with AQHC of the amino termini of peptides purified on the immunoadsorbent was conducted as described previously (*12*).

*Analytical Methods.* Unmodified peptides were purified on a Spectra-Physics SP8100 system for HPLC with a variable wavelength Spectra-Physics Spectra 100 UV−vis detector. All peptides, both unmodified and modified with AQHC, were separated on an analytical C-18 column (0.46 × 25 cm) containing octadecylsilyl silica gel equilibrated with 0.05% trifluoroacetic acid in water and developed with a linear gradient of 1% $CH_3CN$ $min^{-1}$ (*24*). Hydrolyses of peptides were performed in evacuated glass hydrolysis tubes containing 6 M HCl for 45 min at 160 °C. The resulting hydrosylates were analyzed using a modular system composed of a Spectra-Physics autosampler SP8875, Spectra-Physics ternary pump SP8800, and a Pickering sodium cation exchange column. Amino acids were quantified as their ninhydrin derivatives. Densitrometry was performed using a LKB Bromma 2202 Ultroscan.

## RESULTS

*Isolation of the Peptides Containing Cysteines Participating in the Reaction of Ribonucleotide Reductase*. The formation of the cystine between cysteine 225 and cysteine 462 in the pre-steady state has already been followed by quench flow and site-directed immunochemistry (*12*). A similar method was developed to follow the formation of the cystine between cysteine 754 and cysteine 759.

Initial experiments were performed to determine if *S*-(*N*-ethylsuccimidyl) peptides containing cysteine 754 and cysteine 759 could be isolated from proteolytic digests of R1 dimer that had been modified with NEM. The carboxy-terminal sequence of the R1 polypeptide, which contains both cysteine 754 and cysteine 759, is -PSIQDDGCESGACKI. Thermolysin was found to remove the amino-terminal dipeptide PS from the synthetic peptide PSIQDDGCESGACK. Therefore, thermolysin was chosen to cleave on the amino-terminal side of Isoleucine 749 from precipitated R1 dimer. Reduced R1 dimer (10 nmol) was alkylated with a molar excess of NEM. The protein was precipitated with trichloroacetic acid and digested with thermolysin in the presence of NEM. Thermolytic peptides containing the amino-terminal sequence IQDDG- were isolated from the digest by immunoadsorption using an immunoadsorbent made from polyclonal immunoglobulins directed against the amino-terminal sequence of the synthetic peptide IQDDGCE. These peptides isolated by the immunoadsorbent from the digest of alkylated R1 dimer were submitted to HPLC. Two peaks of absorbance at 229 nm were observed with retention times of 21 and 35 min. The amino acid composition following hydrolysis in acid of the fractions associated with the first peak was $I_{0.9}E_{2.2}D_{1.9}G_{2.0}S_{1.0}$, and that of the fractions associated with the second peak was $I_{1.8}E_{2.2}D_{1.9}G_{2.3}S_{0.9}A_{0.9}K_{1.0}$. These compositions indicate that thermolysin cleaved the R1 polypeptide before isoleucine 749 as predicted, but also before alanine 758 to produce the peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESG containing cysteine 754 in addition to the expected peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]KI containing cysteine 754 and cysteine 759.

Additional experiments were performed to isolate thermolytic peptides containing an intact cystine between cysteine 754 and cysteine 759 from RDPR. To form this cystine, prereduced R1 dimer (10 nmol) was mixed with a 2-fold molar excess of R2 dimer in the presence of HEPES buffer containing EDTA and $MgSO_4$, and CDP was added. The reaction was manually quenched after 15 min with a solution of acetic acid containing NEM. The enzyme, oxidized by its substrate, was precipitated with trichloroacetic acid and the precipitate was digested with thermolysin in the presence of NEM. Thermolytic peptides with the amino-terminal sequence IQDDG- were isolated from the digest by immunoadsorption and submitted to HPLC. The main peak of absorbance observed on the chromatogram had a retention time of 25 min. The amino acid composition following hydrolysis in acid of half of the fraction associated with this peak was $I_{1.1}E_{2.1}D_{1.9}G_{1.9}S_{1.3}A_{1.0}K_{1.0}C{-}C_{0.7}$ (where C−C is cystine observed on the chromatogram of the hydrolysate). Another portion of the sample was reduced and alkylated with NEM, the product was submitted to HPLC, and a new peak of absorbance was observed with a retention time (35 min) identical to that of the peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]KI. These data demonstrate that the fraction associated with the peak of absorbance at 25 min in the original sample

was the peptide IQDDGCESGACKI containing an intramolecular cystine. The oxidized peptide was not cleaved before alanine 758.

*Preparation of Peptide Standards*. The two *S*-(*N*-ethylsuccimidyl) peptides were isolated from reduced, alkylated R1 dimer, and the peptide containing the cystine between cysteine 754 and cysteine 759 was isolated from R1 dimer that had been oxidized by its substrate. These peptides were modified with AQHC and used as standards for HPLC. The fluorescent derivatives of the peptides IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESG, IQDDGCESGACKI containing the cystine, and IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)-cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]KI, had retention times of 29, 32, and 36 min, respectively (Figure 1A, left panel). The *N*-ethylsuccinimidylated peptide at 36 min eluted as a doublet presumably because the alkylation produced diastereomers (*12*). The relative yields of the fluorescence for the modified peptides were calculated from the areas of the peaks of fluorescence and the nanomoles of each peptide submitted to chromatography, as determined by quantitative amino acid analysis. The ratio of the yield of fluorescence for the peptide IQDDGCESGACKI containing the peripheral cystine to the yield of fluorescence for the peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESG was 1.3. The ratio of the yield of fluorscence for the peptide, IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]KI to the yield of fluorescence for the peptide IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]-ESG was 2.0.

*Rates for the Formation of the Cystine in the Active Site and the Peripheral Cystine Determined Simultaneously*. It was discovered by isolating peptides containing the peripheral cystine by immunoadsorption that under the same conditions previously chosen to investigate the formation of the cystine between cysteine 225 and cysteine 462 (*12*), the peripheral cysteines were nearly completely oxidized before the initiation of the reaction by the addition of CDP. To reduce the amount of this nonenzymatic oxidation, the pH of the solution containing R1 dimer in the syringe of the quench flow apparatus was reduced to 6.5. Prereduced R1 dimer that was had been treated with hydroxyurea as described by Stubbe[2] to eliminate any contaminating R2 dimer was mixed with CDP and ATP, and loaded into one syringe of the quench flow apparatus. R2 dimer was added to the other syringe, and the effluents from the two syringes were mixed and quenched after the appropriate time with acetic acid and NEM. Control experiments were performed to ensure that the formation of the cystine in the active site after quenching with acetic acid and NEM was negligible. A solution of acetic acid that contained NEM was added simultaneously with substrate to the enzyme. No cystine from the active site could be detected even after overnight incubations of the quenched reaction. If thiol-disulfide exchange were occurring following alkylation with NEM, one would expect to find mixed disulfides between the affinity peptides containing the redox-active cysteines and other peptides containing cysteines on the HPLC following immunoadsorption. No mixed disulfides were discovered other than those containing the expected peripheral and active site disulfides. Control experiments were performed in which R2 protein was omitted and for which a 2 s time point was collected in the quench flow apparatus. The percent cystine

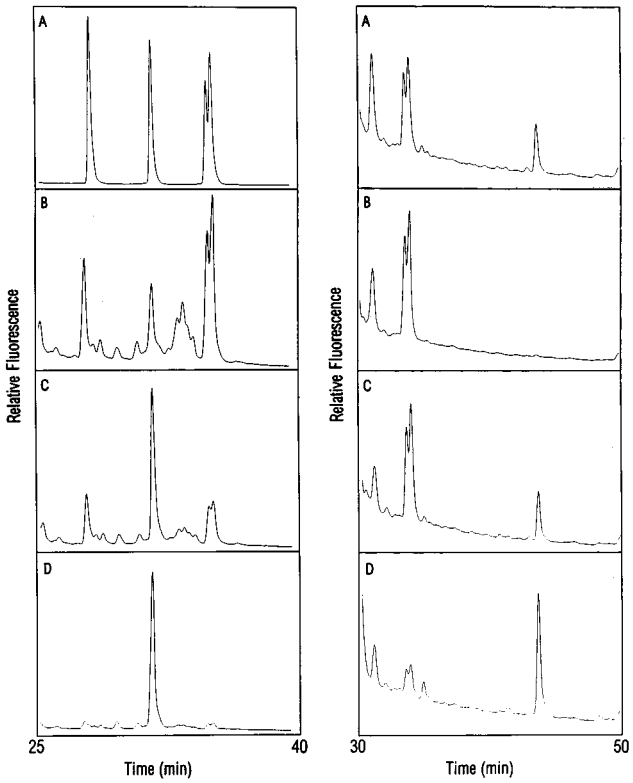



FIGURE 1: Isolation of peptides containing the cysteines oxidized during the pre-steady state from rapidly quenched samples of ribonucleotide reductase. The enzymatic reaction was quenched after the appropriate interval with a solution of acetic acid containing NEM. Protein was precipitated with trichloroacetic acid and split in half. One-half was digested with chymotrypsin and the glutamyl endopeptidase from *S. aureus* strain V8, and the digest was passed over the immunoadsorbent (*12*) specific for peptides with carboxy-terminal sequence -VLIE (2 nmol capacity). The other half was digested with thermolysin and passed over the immunoadsorbent specific for peptides with the amino-terminal sequence IQDDG- (8 nmol capacity). The columns were washed with PBS to remove unbound peptides, and specifically bound peptides were eluted with 0.5 M acetic acid. Acid eluants were evaporated to dryness three times, modified with AQHC, and submitted to reverse phase HPLC on an analytical column (0.46 × 25 cm) of octadecylsilyl silica gel run in 0.05% TFA in water and developed with a linear gradient of 1% $CH_3CN$ $min^{-1}$. The effluent from the column was monitored for fluorescence with an excitation filter with a cutoff of 254 nm and an emission filter with a bandwidth of 370−700 nm. The right panels are chromatograms of peptides containing the cysteines from the active site, and the left panels, peripheral cysteines. (A) A chromatogram of the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE (34 min) and the mixed disulfide between SSCVLIE and IALCTL (43 min) both modified at their amino termini with AQHC (right) and a chromatogram of the peptides IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinly]ESG (29 min), IQDDGCESGACKI containing the peripheral cystine (32 min), and IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)-cysteinyl]KI (36 min) all three modified at their amino termini with AQHC (left). (B) Chromatograms of the respective peptides isolated from ribonucleotide reductase that was mixed in the quench flow apparatus without CDP and reacted 2 s before quenching. (C) Chromatograms of the respective peptides isolated from a sample of ribonucleotide reductase that was mixed in the quench flow apparatus with CDP and reacted for 100 ms before quenching. (D) Chromatograms of the respective peptides isolated from ribonucleotide reductase mixed with CDP and allowed to react for 1 s before quenching.

[2] Personal communication, JoAnne Stubbe, Massachusetts Institute of Technology.

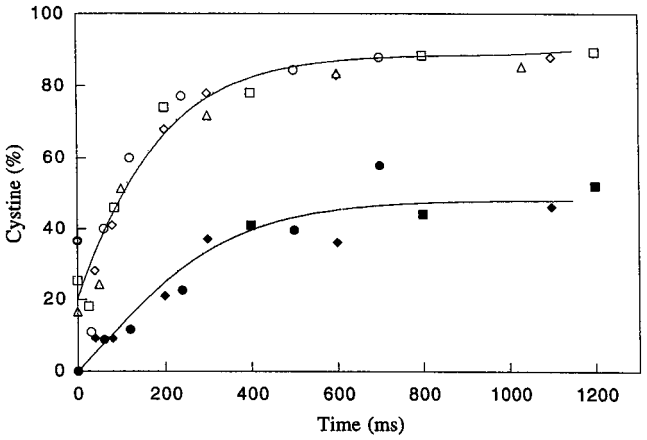


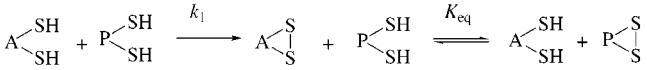

FIGURE 2: Percent of the cystine from the active site and percent of the peripheral cystine formed upon mixing R1 dimer and CDP with R2 dimer as a function of time. Enzyme was mixed with CDP in a quench flow apparatus as described in Figure 1. The areas of the peaks of fluorescence corresponding to the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE, the mixed disulfide between SSCVLIE and IALCTL, and the mixed disulfide between SSCVLIE and CTL (*12*) were measured for each time point from the chromatograms of effluents from the immunoadsorbent (Figure 1, right). These areas and the relative yields of fluorescence for each peptide were used to calculate the percent of cystine from the active site at each time point (closed symbols). The areas of the peaks of fluorescence corresponding to the peptides IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinly]ESG and IQDDG[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]ESGA[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]KI, and the peptide IQDDGCESGACKI containing the intact peripheral cystine were measured for each time point from chromatograms of effluents from the immunoadsorption (Figure 1, left). These areas and the relative yields of fluorescence for each peptide were used to calculate the percent of peripheral cystine at each time point (open symbols). Percent cystine is presented as a function of time (milliseconds). Each set of symbols corresponds to a different experiment. The curves were generated with eq 8.

between cysteine 754 and cysteine 759 in these samples was found to average 23% ± 13% ($n$ = 4). Another control experiment was performed in which CDP was omitted and for which a 2s time point was collected in the apparatus for quench flow. The percent cystine between cysteine 754 and cysteine 759 in this sample was 18%. In a separate experiment, the R1 protein stood at pH 6.5 for up to 30 min and no loss of activity was observed in the steady-state assay.

Once conditions had been found to minimize the adventitious oxidation of the peripheral cysteines, a time course for the enzymatic formation of both the cystine in the active site and the peripheral cystine simultaneously could be run in the quench flow apparatus. The quenched solution for each time point was split in half, and the protein in each sample was precipitated with trichloroacetic acid. The percent cystine between cysteine 225 and cysteine 462 was determined in the one-half of the samples and the percent cystine between cysteine 754 and cysteine 759 was determined in the other half of the samples. The protein in the first half of the samples was digested with chymotrypsin and the glutamyl endopeptidase from *Staphylococcus aureus* strain V8 and the protein in the second half of the samples was digested with thermolysin. The respective peptides containing the redox-active cysteines were purified by immunoadsorption (*25*), and submitted to HPLC (Figure 1B−D). The data from several time courses are displayed in Figure 2.

*Kinetic Mechanism for Ribonucleoside Diphosphate Reductase*. The most peculiar aspect of these results is that the

Scheme 1



peripheral cystine appears to form more rapidly than the cystine in the active site. A kinetic mechanism for RDPR consistent with the observed formation of each cystine in the pre-steady state, but incorporating the requirement that the formation of the cystine in the active site precede the formation of the peripheral cystine is presented in Scheme 1 where the cysteines in the active site, cysteine 225 and cysteine 462, are represented by A and the peripheral cysteines, cysteine 754 and cysteine 759, are represented by P. The rate at which the equilibrium between the cystines is established is so fast relative to $k_1$ that its individual rate constants do not contribute to the overall rates. If

$$(1)\quad [A]_{Tot} = \left[A\begin{smallmatrix}S\\S\end{smallmatrix}\right]_0 + \left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right] + \left[A\begin{smallmatrix}S\\S\end{smallmatrix}\right] + \left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]_\infty$$

$$(2)\quad [P]_{Tot} = \left[P\begin{smallmatrix}S\\S\end{smallmatrix}\right]_0 + \left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right] + \left[P\begin{smallmatrix}S\\S\end{smallmatrix}\right] + \left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]_\infty$$

and

$$(3)\quad [A]_{Tot} = [P]_{Tot} = 100\%$$

then the concentrations of the species actually participating in the pre-steady state are

$$(4)\quad [A]_{part} = [A]_{Tot} - \left[A\begin{smallmatrix}S\\S\end{smallmatrix}\right]_0 - \left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]_\infty = \left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right] + \left[A\begin{smallmatrix}S\\S\end{smallmatrix}\right]$$

$$(5)\quad [P]_{part} = [P]_{Tot} - \left[P\begin{smallmatrix}S\\S\end{smallmatrix}\right]_0 - \left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]_\infty = \left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right] + \left[P\begin{smallmatrix}S\\S\end{smallmatrix}\right]$$

where subscripts 0 indicate initial concentrations and subscripts ∞ indicate final concentration. With these corrections, the rate equation for Scheme 1 could be solved.

$$(6)\quad \frac{d\left(\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right] + \left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]\right)}{dt} = k_1\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]$$

$$(7)\quad K_{eq} = \frac{\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]\left[P\begin{smallmatrix}S\\S\end{smallmatrix}\right]}{\left[A\begin{smallmatrix}S\\S\end{smallmatrix}\right]\left[P\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]}$$

$$(8)\quad \ln\left(\frac{\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]}{[A]_{part}}\right) - \left(\frac{[P]_{Tot}}{K_{eq}[A]_{part}}\right)\ln\left[\frac{\left(Keq[A]_{part} + (1\text{-}K_{eq})\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]\right)}{\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]}\right] + \frac{[P]_{part}}{K_{eq}[A]_{part} + (1\text{-}K_{eq})\left[A\begin{smallmatrix}SH\\SH\end{smallmatrix}\right]} - \frac{[P]_{part}}{[A]_{part}} = -k_1 t$$

The curves displayed in Figure 2 were generated with eq 8 using an Excel spread sheet and values of 1%, 21%, 2.0 $s^{-1}$, 10.3, 48%, and 69% for $[ASS]_0$, $[PSS]_0$, $k_1$, $K_{eq}$, $[A]_{part}$, and $[P]_{part}$, respectively.

It is reasonable to assume that 21% cystine between cysteines 754 and 759 present a zero time ($[PSS]_0$) is technically unavoidable, adventitious oxidation of otherwise competent peripheral cysteines identical in their disposition to the reduced cysteines 754 and 759 present at the beginning of the reaction. Since $K_{eq}$ is 10, the 2% cystine at the active site expected to be in equilibrium with the amount of cystine (21%) at the periphery at zero time is within experimental

error of the amount of cystine (1%) observed in the active site at zero time ($[ASS]_0$). If this assumption is correct, then the reaction is simply initiated in the apparatus for quench flow at the point along its normal trajectory at which it is 23% of the way to completion rather than 0% of the way to completion. If this analysis of the situation is accurate, then the amount of cystine formed in the pre-steady state between cysteine 225 and 462 is 49% and that between cysteine 754 and 759 is 90%.

## DISCUSSION

It was proposed by Lin et al. (*14*) that the R1 protomer contains two pairs of cysteines that participate as reductants in the enzymatic reaction of ribonucleotide reductase. Subsequent studies with mutants of R1 dimer have been consistent with this proposal and led to a proposal for the identity of the participating cysteines (*10*, *11*). Two of these cysteines, cysteine 225 and cysteine 462, participate directly in the reduction of the ribonucleotide by supplying the electron and the hydrogen to the ribonucleotide so that at the completion of one turnover they are present as a cystine (*12*). These two cysteines are connected by electron density in the map for oxidized R1 dimer from *E. coli* (*1*) but are not connected by electron density in the map for reduced R1 dimer (*2*). It should be noted, however, that the oxidized form of the R1 dimer was not produced by the addition of substrate to the reduced form. The recent crystallographic molecular model of the anaerobic ribonucleoside triphosphate reductase from bacteriophage T4 has brought into question the role of the cysteines in the active site in the enzymatic mechanism of ribonucleotide reductase from *E. coli*. The structures surrounding the respective active sites of the two models, the one for the enzyme of bacteriophage T4 and the one for the enzyme from *E. coli*, are superposable, but only one of the two cysteines that are supposed to form the cystine in the active site in the aerobic enzyme from *E. coli*, the cysteine in the enzyme from the bacteriophage homologous to cysteine 225, is conserved in the active site of this enzyme (*26*). The fact, however, that the formation of the cystine in the active site of RDPR from *E. coli* is kinetically competent (*12*) demonstrates that the formation of this cystine is involved in turnover of ribonucleotide reductase.

The other pair of cysteines implicated in the mechanism of ribonucleotide reductase is located near the carboxy terminus of an R1 protomer at positions 754 and 759 in the enzyme from *E. coli*. These cysteines are thought to shuttle reducing equivalents from reduced thioredoxin or glutaredoxin to the cysteines in the active site by disulfide interchange (*10*, *13*, *14*). Formation of the cystine between cysteine 754 and cysteine 759, however, has never before been observed directly, and the carboxy terminus that contains the peripheral cysteines is not present in the maps of electron density of either the oxidized or reduced proteins. Support for the role of the peripheral cysteines has come mainly from studies of site-directed mutants (*10*, *13*). Berardi et al. (*27*) have prepared a mixed disulfide between a mutant of glutaredoxin from *E. coli* and a peptide corresponding to the last 25 amino acids of ribonucleotide reductase from *E. coli*. In this peptide, the cysteine analogous to cysteine 759 from ribonucleotide reductase performed the role of the electrophile in disulfide interchange with reduced glutaredoxin. These results provide additional indirect support for the proposed role of the peripheral cysteines in the transfer of reducing equivalents from glutaredoxin to the ribonucleotide.

The data for the kinetics of the formation of cystine in the active site during the pre-steady state was fit to eq 8 derived from Scheme 1 using the value of 2.0 $s^{-1}$ for $k_1$. In the earlier study (*12*), the first-order rate constant for the formation of the cystine in the active site determined under the same conditions as those of the present study was 2.6 $s^{-1}$, which agrees well with the present value. In addition, the observation in the present study that only 50% of the cysteines in the active sites participated in the burst of cystine formation seen in the pre-steady state is also consistent with the earlier ones.

One unexpected result in these present experiments is that in the pre-steady state, all of the peripheral cysteines (90%) become cystines while only half of the cysteines (49%) in the active sites become cystines (Figure 2). It follows from this observation either that peripheral cysteines from more than one protomer can service the same active site or that peripheral cysteines engage in rapid disulfide interchange among themselves. Such disulfide interchange between two pairs of peripheral cysteines on the same R1 dimer would be reminiscent in a small way of the 72 lipoic acids that are able to rapidly exchange acetyl groups among themselves in pyruvate dehydrogenase (*28*, *29*). It is also possible, however, that disulfide interchange among peripheral cysteines could take place intermolecularly between R1 dimers. Although the pathways involved were not determined, Mao et al. (*9*) found that R1 dimers of a catalytically inactive mutant were able to transfer reducing equivalents to wild type R1 dimers but more slowly than can thioredoxin. Pre-steady state studies of this necessarily interdimeric disulfide interchange were not performed. The equilibration among the peripheral cystines observed in the present studies was much more rapid than the formation of cystine at the active site (2 $s^{-1}$), and this rapidity suggests that the disulfide interchange observed here takes place within an R1 dimer rather than between R1 dimers.

The crystallographic molecular models of the R1 dimer and the R2 dimer show that each of the two proteins have C2 symmetry. It follows that within a free, unassociated R1 dimer if the peripheral cysteines of one protomer have access to one of the active sites on the dimer, the peripheral cysteines on the other protomer necessarily have access to the other active site on the dimer. During the presteady state, the cysteines in only half of the active sites on R1 protomers become cystines while all of the peripheral cysteines become cystine (Figure 2). If the cysteines in the active sites that do not become cystines had been able to participate in disulfide interchange with peripheral cystines as the cysteines in active sites that do become cystines must do, then the enzymatic reaction at the active sites that are functional would necessarily have driven the formation of cystine at all of the active sites. If the complex between R1 dimer and R2 dimer were C2 symmetric as are both R1 dimer and R2 dimer that compose it, all of the cysteines in the active sites would have had to become cystines if all of the peripheral cysteines become cystine, but they did not. It follows that the active complex between R1 dimer and R2 dimer cannot be symmetric and that in the complex, one of the active sites is no longer connected to the peripheral cysteines while the

other remains so. The most logical explanation for this disconnection is that the structure of the complex is such that it sterically prevents peripheral cystines from entering one of the two active sites within an R1 dimer and oxidizing the cysteines in that active site. Regardless of the detailed mechanism, the functional complexes between R1 dimers and R2 dimers cannot have the C2 symmetry of their components.

One explanation consistent with the observation that in the pre-steady state, cystines are formed in only half of the active sites on R1 protomers (Figure 2) is that at any time only one of the two active sites in each R1 dimer can be coupled to a tyrosyl radical in an R2 dimer because of the asymmetry of the functional complex between R1 dimers and R2 dimers. This asymmetry, in addition to blocking access of the peripheral cysteines to one of the two active sites on an R1 dimer in the complex, would also prevent that same active site from participating in an effective connection to the tyrosyl radical in the protomer of an R2 dimer. Such an asymmetric arrangement has been described for connections among the heteroprotomers of 2-oxoglutarate dehydrogenase (*30*).

Two other unexpected results were observed in the present experiments. First, the value obtained for $K_{eq}$ suggests that the peripheral cystine has a lower redox potential than the cystine in the active site. This property would make sense if the role of the peripheral cysteines is to keep the active site maximally reduced. This value for $K_{eq}$ would also explain why in the previous experiments that investigated the formation of the cystine in the active site (*12*), the peripheral cysteines could be almost fully oxidized while the cysteines in the active site were almost completely reduced. Second, disulfide interchange between the cysteines in the active site and those at the periphery is more rapid than the reduction of nucleotide in the active site.

In the previous experiments (*12*), it was shown that the rate at which cystine in the active site can be formed is much faster than the rate at which deoxyribonucleotides are formed and that the rate-limiting step of the enzymatic reaction must occur after the formation of the cystine in the active site. Since the rate for the formation of the cystine at the periphery is rapid, the rate-limiting step of the overall reaction catalyzed by ribonucleotide reductase must occur after the formation of the peripheral cystine.

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