Formation of the Cystine between Cysteine 225 and Cysteine 462 from Ribonucleoside Diphosphate Reductase Is Kinetically Competent†

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ABSTRACT: Participation of the formation of the cystine between cysteine 225 and cysteine 462 in the R1 protein to the enzymatic mechanism of aerobic ribonucleoside diphosphate reductase from *Escherichia coli* has been examined by use of rapid quenching and site-directed immunochemistry. Prereduced ribonucleotide reductase in the presence of ATP was mixed with CDP in a quench flow apparatus. The reaction was terminated with a solution of acetic acid and *N*-ethylmaleimide. The protein was precipitated and digested with chymotrypsin and the proteinase from *Staphylococcus aureus* strain V8 in the presence of *N*-ethylmaleimide to yield the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE containing cysteine 225 and the mixed disulfide between the peptide SSCVLIE and the peptide IALCTL containing cysteine 462. These two peptides were retrieved together from the digest by immunoadsorption. The affinity-purified peptides were modified at their amino termini with the fluorescent reagent 6-aminoquinolyl-*N*-hydroxysuccimidyl carbamate and submitted to high-pressure liquid chromatography. The areas of the respective peaks of fluorescence corresponding to the *S*-(*N*-ethylsuccimidyl) peptide, and the mixed disulfide were used to determine the percentage of the cystine that had formed during each interval. The rate constant for the formation of the cystine following the association of free, fully reduced ribonucleotide reductase with the reactant CDP was 8 s^{-1}. Because only 50% of the active sites participated in this pre-steady-state reaction, the maximum steady-state rate consistent with the involvement of this cystine in the enzymatic reaction would be 4 s^{-1}. Since the turnover number of the enzyme under the same conditions in a steady state assay was only 1 s^{-1}, the formation of the cystine between these two cysteines is kinetically competent.

Ribonucleotide reductases catalyze the conversion of ribonucleotides to deoxyribonucleotides. The balanced supply of deoxyribonucleotides produced by this enzyme is necessary for the synthesis and repair of DNA. Ribonucleotide reductases have been variously divided into three (*1*) or four (*2*) classes based on their requirements for cofactors. It is believed, however, that the enzymes in all of the classes use a common mechanism involving free radicals and hydrogen abstractions to effect reduction of the ribonucleotide (*3*−*5*). The ribonucleoside diphosphate reductase from *Escherichia coli* is the prototype for the group of these enzymes usually assigned to class 1 (*1, 2*). Included in this class are the mammalian ribonucleoside diphosphate reductases. Two folded polypeptides, designated R1 and R2, respectively, combine in an as yet undefined stoichiometry to form, in the presence of magnesium, active enzyme (*6, 7*). In the enzyme from *E. coli*, the R1 and R2 polypeptides contain 761 and 375 amino acids, respectively (*8, 9*). The native R1 protein and the native R2 protein purify separately, both as dimers, each dimer containing two folded copies of the respective polypeptide. Each native R1 monomer contains an active site at which all four ribonucleotides are reduced (*10*). The R1 dimer contains allosteric sites that regulate substrate specificity and enzymatic activity (*10*). Each native R2 monomer contains a binuclear iron cluster that forms and stabilizes a free radical located on tyrosine 122 (*11*). Crystallographic molecular models are available for the native R1 dimer (*12, 13*) and the native R2 dimer (*14*) but not for any complex of the two.

A mechanism for ribonucleoside diphosphate reductase has been proposed (*3, 4, 15, 16*). In this proposed mechanism, catalysis is initiated by the binding of a ribonucleotide to the reduced R1 protein, which results in the transfer of an electron from the conjugate base of cysteine 439 on the R1 protein from *E. coli* to the radical centered on tyrosine 122 on the R2 protein. The resulting thiyl radical at cysteine 439 is adjacent to the 3′ position of the ribonucleotide and abstracts a hydrogen from the 3′ carbon to form a 3′ ribonucleotidyl radical. This radical then loses the hydroxyl at the 2′ position by an α-elimination that is accomplished by the removal of the proton from the 3′ hydroxyl coincident with the protonation of the 2′ hydroxyl. The driving force for this elimination is the formation of a stable oxallylic radical. The 2′ carbon of the oxallylic radical abstracts a hydrogen from cysteine 225 located on the same side of the ribose ring of the ribonucleotide from which the 2′ hydroxyl just left to form the 2′-deoxy-3′-oxoribonucleotide. The resulting cysteinyl radical then forms a cystine radical anion with the conjugate base of cysteine 462. The 3′ oxygen of the 2′-deoxy-3′-oxoribonucleotide is protonated, and the cystine radical anion, becoming in the process a cystine between cysteine 225 and cysteine 462, transfers an electron

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to the 3′ carbon of the protonated ketone to form a radical on the 3′ carbon. Turnover is complete when this radical at the 3′ carbon reabstracts the hydrogen from cysteine 462, the same cysteine that initiated the process, returning it to a thiyl radical. The cystine between cysteine 225 and cysteine 462 must be rereduced for the enzyme to complete additional turnovers. This rereduction for enzymes of class 1 and 2 is thought to occur by disulfide exchange with two cysteines located near the carboxy terminus of the R1 protein (*17*−*19*). The cystine formed between cysteine 754 and cysteine 759 would then be reduced by either reduced thioredoxin or reduced glutaredoxin (*20*, *21*).

Thelander (*7*) and Lin et al. (*19*) have shown that, over a period of 5−10 min, 1 mol of the prereduced R1 dimer of ribonucleoside diphosphate reductase (2 mol of active sites) in the presence of 0.1−2 mol of active R2 dimer but in the absence of external reductant will convert 3 mol of CDP to dCDP. Concomitant with the formation of dCDP, 6 mol of cysteine within the mole of R1 dimer was oxidized to 3 mol of cystine. Additional studies with site-directed mutants support the proposal that cysteine 225 and cysteine 462 on the R1 protein are the two cysteines that directly supply the hydrogen atom and the electron required for the reduction of the ribonucleotide (*18*, *22*) and consequently become the cystine immediately adjacent to the product. Also consistent with this proposal is the fact that cysteine 225 and cysteine 462 are connected by electron density in the map of electron density for oxidized R1 dimer (*12*) but are not connected by electron density in the map of electron density for reduced R1 dimer (*13*). The crystallographic molecular model for the reduced form of the enzyme has GDP bound, and in this model these two cysteines are positioned next to the proper face of the ribonucleotide. Although the cystine observed in the oxidized form of the R1 dimer used for the crystallography was not formed enzymatically, its existence demonstrates the ability of this pair of cysteines to form a disulfide. A crystallographic molecular model of the anaerobic ribonucleoside triphosphate reductase from bacteriophage T4 has recently been presented (*23*). Although this enzyme has no statistically significant sequence similarity to the aerobic ribonucleoside diphosphate reductase from *E. coli* and uses a glycyl radical to initiate the reaction rather than a tyrosyl radical, the structures of the two proteins surrounding the respective active sites are superposable. Only one of the two cysteines, however, that are supposed to form the cystine 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. Therefore, the formation of a cystine between cysteine 225 and cysteine 462, a step thought to be essential for the turnover of the aerobic enzyme from *E. coli*, cannot occur during the turnover of the anaerobic enzyme from bacteriophage T4. This observation raises the question of whether the formation of this cystine occurs during turnover of any of the ribonucleotide reductases. To examine more critically the role of cysteine 225 and cysteine 462 in the mechanism of the aerobic enzyme from *E. coli*, I have investigated the rate of their oxidative conjunction during a single turnover by directly isolating a peptide containing the intact cystine between these two cysteines from samples of prereduced ribonucleoside diphosphate reductase rapidly quenched after mixing it with CDP.

EXPERIMENTAL PROCEDURES

Materials. The R1 dimer (ϵ_{272} 190 mM^{-1} cm^{-1}) of *E. coli* ribonucleoside diphosphate reductase with a specific activity of 1.3−1.6 μmol min^{-1} mg^{-1} was isolated from the overproducing strain K38pMJ1 (*24*). The R2 dimer (ϵ_{277} 149 mM^{-1} cm^{-1}) of *E. coli* ribonucleoside diphosphate reductase with a specific activity of 6 μmol min^{-1} mg^{-1} was purified from the overproducing strain C600pMB1 (*25*). The absorption maxima and molar extinction coefficients for the two proteins were determined from scans of the absorbance as a function of wavelength performed on a recording spectophotometer. The concentrations of protein (milligrams per milliliter) of the samples scanned were assayed by quantitative amino acid analysis. The 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. Thioredoxin from *E. coli* with a specific activity of 38 units mg^{-1} was isolated from overproducing strain SK6517 (*26*), and thioredoxin reductase with a specific activity of 240 units mg^{-1} was isolated from strain A326/pTrR301 (*27*, *28*). A unit is defined as a change of one absorbance unit at 412 nm, the wavelength of maximum absorbance for the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] per minute used in the colorimetric assay (*29*). N^{α}-Fluorenylmethyloxycarbonyl (N^{α}-FMOC) amino acids, N^{α}-*tert*-butoxycarbonyl (*t*-BOC) amino acids, 1-hydroxybenzotriazole hydrate, and *p*-alkoxybenzyl solid phase was purchased from either Bachem or Calbiochem−Novabiochem. 6-Aminoquinolyl-*N*-hydroxysuccimidyl carbamate (AQHC) was purchased from Waters Corp. Tris(hydroxymethyl)aminomethane (Tris) was purchased from Fisher Scientific. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), Sephadex G-25, phenylmethanesulfonyl fluoride, *N*-ethylmaleimide (NEM), dithiothreitol (DTT), nucleotides, pyruvate kinase, phosphoenolpyruvate (PEP), and NADPH were purchased from Sigma Chemical Co. Proteinase from *Staphylococcus aureus* strain V8 and chymotrypsin were purchased from Worthington. 1,3-Diisopropylcarbodiimide, *N*-methylpyrrolidinone, ethanedithiol, hydrindantin, and piperidine were purchased from Aldrich Chemical Co. Trifluoroacetic acid (TFA) was purchased from Halocarbon Products. Ninhydrin and dimethylpimilimidate were both purchased from Pierce Chemical Co. Protein A−Affi-Gel was purchased from Bio-Rad Laboratories. 5,5′-Dithiobis-(2-nitrobenzoic acid) was purchased from Calbiochem−Novabiochem.

Assay of Enzymatic Activity. The continuous coupled enzyme assay of Thelander et al. (*30*) was used to determine the steady-state activity of R1 and R2 proteins. The assay was slightly modified to determine the steady-state activity of R1 protein at the concentrations under which the quench flow measurements were performed. The final concentrations

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; AQHC, 6-aminoquinolyl-*N*-hydroxysuccimidyl carbamate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; *t*-BOC, *tert*-butoxycarbonyl; FMOC, 9-fluorenylmethyloxycarbonyl; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; C-18, octadecylsilyl silica gel; PBS, 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4; EDTA, ethylenediaminetetraacetic acid; PEP, phosphoenolpyruvate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

in these assays were 0.5−15 μM R1 dimer, 2.5−75 μM R2 dimer, 40 μM thioredoxin, 1 μM thioredoxin reductase, 15 mM $MgSO_4$, 3 mM ATP, 25 mM PEP, 0.04 mg mL^{-1} pyruvate kinase, 1 mM NADPH, 1 mM CDP, and 50 mM HEPES, pH 7.6. In all cases, initial rates were determined from the initial linear portion of the continuous curves of the change in absorbance against time produced by the recording spectrophotometer. For assays with 15 μM R1 dimer, the rate was calculated from the linear change in absorbance observed in the first 5 s of the reaction.

Synthesis of Peptides. The peptide *N*-acetylRQFSSCVLIE was synthesized on the solid phase using the fluorenylmethyloxycarbonyl strategy (*31*). The O^{γ}-butyl ester of N^{α}-FMOC-L-glutamic acid was attached to a *p*-alkoxybenzyl solid phase with diisopropylcarbodiimide. The preformed hydroxybenzotriazole esters of N^{α}-FMOC-L-isoleucine, N^{α}-FMOC-L-leucine, N^{α}-FMOC-L-valine, N^{α}-FMOC-*S*-(triphenylmethyl)-L-cysteine, N^{α}-FMOC-O^{β}-butyl-L-serine, N^{α}-FMOC-L-phenylalanine, N^{α}-FMOC-L-glutamine, and N^{α}-FMOC-N^{δ}-[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-L-arginine were then added sequentially to complete each cycle of the synthesis. Acetylation of the final, deprotected α-amino group was performed in 1-methyl-2-pyrrolidinone containing 4 equiv of acetic anhydride and 2 equiv of *p*-(dimethylamino)pyridine. The completed product, *N*-acetylRQFSSCVLIE, was cleaved from the solid phase, and all protecting groups were removed by treatment with 90:5:5 TFA/ethanedithiol/thioanisole for 8 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 peptides were purified by reverse-phase, high-pressure liquid chromatography (HPLC).

The peptide IALCTLSAF was synthesized on solid phase by the *tert*-butyloxycarbonyl strategy (*31*). N^{α}-*tert*-Butyloxycarbonyl-L-phenylalanine was coupled to chloromethylpolystyrene−divinylbenzene copolymer, and the *t*-BOC-protected amino acids N^{α}-*t*-BOC-L-alanine, N^{α}-*t*-BOC-O^{β}-benzyl-L-serine, N^{α}-*t*-BOC-L-leucine, N^{α}-*t*-BOC-O^{β}-benzyl-L-threonine, N^{α}-*t*-BOC-L-cystine, N^{α}-*t*-BOC-L-leucine, N^{α}-*t*-BOC-L-alanine, and N^{α}-*t*-BOC-L-isoleucine were then sequentially coupled. The product, the mixed disulfide of IALCTLSAF and IALC, was cleaved from the solid phase by bubbling anhydrous hydrobromic acid through the suspension in 95:2.5:2.5 TFA/anisole/phenol, and following reduction with DTT, the peptide IALCTLSAF was purified by HPLC.

Peptides were hydrolyzed in 6 M HCl for 45 min at 160 °C, and their amino acid compositions were 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 both treatments was in agreement with the expected values for each synthetic peptide.

Synthesis of SS[S-(N-Ethylsuccinimid-2-yl)cysteinyl]VLIE. The synthetic peptide *N*-acetylRQFSSCVLIE was digested with chymotrypsin to remove the amino-terminal tripeptide *N*-acetylRQF. The digest was sparged with argon, treated with 4 mM DTT for 15 min at room temperature, and acidified with acetic acid (5%), and the resulting peptide SSCVLIE was purified by HPLC. Pure, reduced SSCVLIE at 1 mM was modified with 5 equiv of NEM in 0.5 M Tris-HCl, pH 8.0, for 5 min at room temperature. The product SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE was purified by HPLC.

Synthesis of the Mixed Disulfide between SSCVLIE and IALCTL. The synthetic peptide IALCTLSAF was dissolved at 0.5 mM in 1 mM DTNB and 0.1 M Tris-HCl, pH 8.0, and allowed to react for 5 min to form the activated mixed nitrobenzoatyl disulfide. The peptide SSCVLIE was then added to 1 mM. After 30 min at room temperature, the sample was acidified with acetic acid, and the mixed disulfide between SSCVLIE and IALCTLSAF was purified by HPLC. This mixed disulfide was digested with chymotrypsin to remove the carboxy-terminal tripeptide SAF, and the mixed disulfide between SSCVLIE and IALCTL was purified by HPLC. Its identity was confirmed by amino acid analysis.

Preparation of the Immunoadsorbent. Antisera from rabbits immunized with the haptenic conjugate of the peptide KVLIE and BSA (*32*) was passed through an affinity adsorbent prepared by attaching KVLIE to succinylated agarose activated with *N*-hydroxysuccinimide (*32*). The adsorbent was washed with 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4 (PBS), and bound immunoglobulins were eluted with 0.1 M sodium phosphate, pH 2.5. The immunoglobulins were pooled, dialyzed against PBS, concentrated to 1.5 mL, and coupled with dimethylpimilimidate to beaded agarose to which protein A had been attached (*33*). The immunoadsorbent was packed into a column (1 cm × 15 cm) and washed with PBS. The amount of peptide that the immunoadsorbent was capable of binding was determined by passing the peptide KVLIE (20 nmol) over the column, washing with PBS, and eluting the bound KVLIE with 0.5 M acetic acid. The eluted fractions were submitted to reverse-phase HPLC, and the fraction containing the peak of absorbance corresponding to that of the peptide KVLIE was hydrolyzed and subjected to amino acid analysis. The immunoadsorbent had a capacity sufficient to bind 2 nmol of the peptide.

Quench Flow Measurements. Quench flow measurements were performed on a KinTek RGF-3 quench flow instrument. All solutions as well as the instrument were flushed with argon. Prereduced R1 protein (*7*, *19*) dissolved in 15 mM $MgSO_4$, 1 mM EDTA, and 50 mM HEPES, pH 7.6, was distributed into separate tubes, one for each time point. A 5-fold molar excess of R2 protein was added to each tube, and the tubes were sealed under argon and placed on ice. One sample syringe of the quench flow apparatus was loaded with 2 mM CDP, 15 mM $MgSO_4$, 1 mM EDTA, and 50 mM HEPES, pH 7.6. A sealed tube of R1 and R2 proteins was brought to room temperature and mixed with a solution of ATP, PEP, and pyruvate kinase and loaded into the other sample syringe of the quench flow apparatus. Each time point was collected no later than 5 min after mixing the ATP with the enzyme. Samples were quenched in the apparatus with 70% acetic acid containing 20 mM NEM. Two runs were collected for each time point and were mixed together. After each run, the apparatus was flushed with water and ethanol and then sparged with argon. After each quenched sample was removed from the apparatus, four volumes of 8% TCA was added to precipitate the protein. The precipitate was sedimented by centrifugation, and the supernatant was removed by aspiration.

Enzymatic Digestions and Immunoadsorption. To liberate the desired peptides from precipitated ribonucleoside diphosphate reductase, samples were digested with the proteinase from *S. aureus* strain V8 (0.06 mg mL^{-1}) in 4 mM NEM and 50 mM ammonium bicarbonate, pH 7.8, for 2 h at 37 °C, followed by digestion with 0.1 mg mL^{-1} chymotrypsin for 2 h at 37 °C. Another aliquot of the proteinase from *S. aureus* strain V8 was added to 0.06 mg mL^{-1}, and the sample was digested for 1 h at 37 °C followed by a final digestion with 0.1 mg mL^{-1} chymotrypsin for 1 h at 37 °C. Chymotrypsin was inhibited with 5 mM phenylmethanesulfonyl fluoride, and the digests were passed over the immunoadsorbent. The immunoadsorbent was washed with 45 mL of PBS, and those peptides that were specifically bound 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 phosphate, pH 2.5; 15 mL of 0.5 M acetic acid; 15 mL of 0.1 M phosphate, pH 2.5; and 15 mL of PBS.

Fluorescent Modification of Affinity-Purified Peptides. The pool of an acidic eluate (3 mL) from the immunoadsorption was evaporated in a centrifugal vacuum concentrator (Savant Instruments, Inc.), and the residue was redissolved in 150 μL of 50% acetonitrile. Each sample was transferred to an Eppendorf tube, evaporated, redissolved in 100 μL of water, and reevaporated. Each of the final residues was dissolved in 100 μL of 0.2 M borate buffer, pH 8.8. 6-Aminoquinolyl-*N*-hydroxysuccimidyl carbamate (2 mM) was added, and the Eppendorf tubes were vortexed. The samples were heated to 55 °C for 5 min and then acidified with 25 μL of 0.5 M acetic acid. The AQHC derivatives of the peptides were separated on a reverse-phase HPLC system consisting of a Waters 600E solvent delivery system equipped with a Waters WISP model 710V auto injector, Laboratory Data Control fluorometer III, and a Nelson model 444 data collection system. The fluorescence of the effluent was monitored with a 254 nm excitation filter and a 370−700 nm emission filter.

Analytical Methods. Unmodified peptides were purified on a Spectra-Physics chromatography SP8100 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 cm × 25 cm) run in 0.05% TFA in water and developed with a linear gradient of 1% CH_3CN min^{-1}. Hydrolyses of peptides and proteins were performed in evacuated glass hydrolysis tubes containing 6 M HCl for 45 min at 160 °C. The resulting hydrosylates were analyzed on 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. Densitometry was performed with a LKB Bromma 2202 Ultroscan.

RESULTS

Proteolytic Digestion To Produce the Intact Mixed Disulfide between the Peptides SSCVLIE and IALCTL. In a previous study (*32*), an immunoadsorbent had been constructed to retrieve peptides containing the carboxy-terminal sequence -VLIE, corresponding to the amino acid sequence of R1 protein immediately following cysteine 225. This immunoadsorbent was to be used in the present studies to determine the amount of the cystine formed between cysteine 225 and cysteine 462 in a given preparation of R1 protein by isolating a peptide fragment containing this cystine intact. Cysteine 225 is found in the sequence -RQFSSCVLIECGD-SL- between arginine 220 and leucine 234, and cysteine 462 is found in the sequence -ENGEIALCTLSAFNLGAIN- between glutamate 455 and asparagine 473. The ability of the proteinase from *S. aureus* strain V8 to cleave the polypeptide chain of the R1 protein after glutamate 230 had already been demonstrated (*19*). It was expected that the proteinase from *S. aureus* strain V8 would also cleave adjacent to glutamate 458. Another proteinase was needed to cleave the polypeptide on the amino-terminal side of cysteine 225 and the carboxy-terminal side of cysteine 462 while leaving the carboxy-terminal sequence -VLIE intact. The peptides *N*-acetylRQFSSCVLIE and IALCTLSAF were synthesized to use for test digestions and to prepare standards.

Chymotrypsin cut the synthetic peptide *N*-acetylRQFSS-CVLIE cleanly after the phenylalanine to yield the peptide SSCVLIE. Additional digestions using a 10-fold greater concentration of chymotrypsin were performed overnight with the synthetic peptide KVLIE, and it was found that even under these more severe conditions the epitope -VLIE was not cleaved by the chymotrypsin. The peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE was prepared synthetically from the peptide *N*-acetylRQFSSCVLIE for use as a standard on HPLC. The synthetic product ran as a doublet on the chromatogram. A additional set of experiments suggested that this doublet was produced from the two diastereomers of the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)-cysteinyl]VLIE. The fractions containing each of the peaks of the doublet were submitted to amino acid analysis following acid hydrolysis. The amino acid composition of the fraction containing the first peak was $S_{1.6}V_{1.0}L_{1.1}I_{1.0}E_{1.4}$ and that of the fraction containing the second peak was $S_{1.7}V_{1.0}L_{1.1}I_{1.0}E_{1.3}$. It was found that when reduced R1 protein was modified with *N*-ethylmaleimide, precipitated with TCA, and digested with the proteinase from *S. aureus* V8 and chymotrypsin, a doublet of absorbance that coincided with that of the standard peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)-cysteinyl]VLIE could be isolated reproducibly by immunoadsorption and HPLC. A portion of each of the two fractions associated with each of the two peaks of absorbance of the doublet that had been isolated from R1 protein were submitted to electrospray mass spectrometry. Each of the two samples had molecular ions of the same mass (*m*/*z* of 875.2 and 875.3). A pool of the two peaks gave the amino acid composition $S_{1.6}V_{1.1}L_{1.0}I_{1.0}E_{1.3}$ as expected. These results suggest that the two peptides in the doublet are the diastereomers of SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE.

To test whether chymotrypsin could cleave on the carboxy-terminal side of cysteine 462, a mixed disulfide was prepared from the synthetic peptides SSCVLIE and IALCTLSAF. The synthetic peptide was digested with chymotrypsin and submitted to HPLC. A peak of absorbance at 229 nm was observed with a retention time of 38 min. The amino acid composition of the fraction associated with this peak following acid hydrolysis was $S_{1.8}V_{1.2}L_{3.0}I_{1.9}E_{1.1}A_{1.0}T_{1.0}$, demonstrating that chymotrypsin cleaves between leucine and serine to yield the mixed disulfide between the peptide SSCVLIE and the peptide IALCTL. A peptide with the same retention time as the synthetic mixed disulfide could be

purified by immunoadsorption from R1 protein that had been partially oxidized by mixing it at 125 mM with R2 dimer (12.5 mM), ATP (1.6 mM), and CDP (1 mM), precipitated with trichloroacetic acid, and digested with chymotrypsin and the proteinase from *S. aureus* V8. The amino acid composition of this peptide was $S_{1.6}V_{1.2}L_{3.2}I_{1.9}E_{1.1}A_{1.1}T_{0.9}$.

Fluorescent Modification of Affinity-Purified Peptides. The synthetic standards for the mixed disulfide between SSCVLIE and IALCTL and the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE were modified at their amino termini with the fluorescent reagent AQHC to yield fluorescent derivatives. These modified standard peptides could be detected on HPLC by monitoring the fluorescence of the effluent with a fluorometric detector equipped with a 254 nm excitation filter and a 370−700 nm emission filter (Figure 1A,B). A doublet thought to be due to the diastereomers of SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE was again seen (Figure 1A). The fluorescent modification increased the sensitivity of the chromatographic separation of these peptides 50-fold and allowed the experiments to be performed with less enzyme at lower concentrations. The relative yields of the fluorescence for the adduct between AHQC and the mixed disulfide of SSCVLIE and IALCTL (43 min) and that between AHQC and the peptide SS[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE (34 min) were calculated. The ratio of the yield of fluorescence for the synthetic standard for the mixed disulfide to the yield of fluorescence for the synthetic standard for the alkylated peptide was 2.1:1 as expected theoretically. The percentage of cysteine 225 present as a cystine with cysteine 462 was calculated for each sample submitted to this analysis from the areas of the peaks of fluorescence and the relative yields of fluorescence.

Rate of Formation of the Cystine between Cysteine 225 and Cysteine 462 in the Presence of ATP, PEP, and Pyruvate Kinase. The sensitivity with which the fluorescently labeled peptides could be detected allowed the experiments in the quench flow apparatus to be performed with 3 μM R1 dimer. When this concentration of R1 protein was assayed with 15 μM R2 dimer, 1 mM CDP, and 3 mM ATP, the steady-state turnover number was 1.1 μmol of oxidized thioredoxin (μmol of active site)$^{-1}$ s^{-1}. In the experiments examining single turnovers, one syringe of the quench flow apparatus was loaded with ATP, R1 protein, R2 protein, and HEPES buffer containing EDTA and $MgSO_4$. The other syringe was loaded with CDP and HEPES buffer containing EDTA and $MgSO_4$. Upon mixing of the effluents from the two syringes, the concentrations of all components in the following experiments were the same as those used in the steady-state assay. Samples were quenched in the apparatus by mixing them with a solution of acetic acid containing NEM.

In an initial control experiment, two conditions were examined. The first was a sample from which CDP was omitted and for which a 1 s time point was collected from the quench flow apparatus. Analysis of the recovered peptides following digestion, immunoadsorption, fluorescent modification, and HPLC revealed the percent cystine formed between cysteine 225 and cysteine 462 in this sample to be 70%. The second was a sample from which both ATP and CDP were omitted and for which a 1 s time point also was collected. The percent cystine formed between cysteine 225 and cysteine 462 in this second sample was found to be less than 5%. The high percent cystine found in the sample

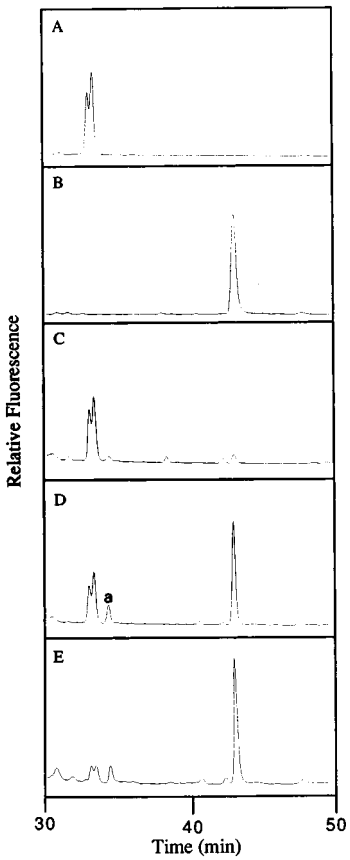

FIGURE 1: Isolation of peptides containing cysteine 225 from rapidly quenched samples of ribonucleoside diphosphate reductase. Prereduced R1 protein, R2 protein, ATP, pyruvate kinase, PEP, and HEPES buffer containing $MgSO_4$ and EDTA were added to one syringe of a quench flow apparatus. Cytidine diphosphate and HEPES buffer containing $MgSO_4$ and EDTA were added to the other syringe. The final concentrations upon mixing the effluents from the two syringes were 3 μM R1 dimer, 15 μM R2 dimer, 3 mM ATP, 25 mM PEP, 0.04 mg mL^{-1} pyruvate kinase, 1 mM CDP, 15 mM $MgSO_4$, 1 mM EDTA, and 50 mM HEPES, pH 7.6, in a final volume of 40 μL for each run. The enzymatic reaction was quenched after the appropriate interval with a solution of acetic acid containing NEM, and two runs were pooled for each time point. Protein was precipitated with trichloroacetic acid and digested with chymotrypsin and the proteinase from *S. aureus* strain V8. The digest was passed over the immunoadsorbent specific for peptides with the carboxy-terminal sequence -VLIE (2 nmol capacity). The column was 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 C-18 column (0.46 cm × 25 cm) 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 a 254 nm excitation filter and a 370−700 nm emission filter. For all chromatograms, fluorescence is presented as a function of time of elution (minutes). (A) Chromatogram of the synthetic peptide SS-[*S*-(*N*-ethylsuccinimid-2-yl)cysteinyl]VLIE modified at its amino terminus with AQHC. (B) Chromatogram of the synthetic mixed disulfide between SSCVLIE and IALCTL modified at its amino termini with AQHC. (C) Chromatogram of peptides isolated from ribonucleoside diphosphate reductase that was mixed in the quench flow apparatus without CDP and reacted for 2 s before quenching. (D) Chromatogram of peptides isolated from a sample of ribonucleoside diphosphate reductase that was mixed in the quench flow apparatus with CDP, and reacted for 40 ms before quenching. Peak a was found to be the mixed disulfide between the peptide SSCVLIE and the peptide CTL modified at its amino terminus with AQHC. (E) Chromatogram of peptides isolated from ribonucleoside diphosphate reductase mixed with CDP and allowed to react for 1 s before quenching.

containing ATP and no CDP was thought to result from turnover by the enzyme of small amounts of contaminating ADP, a substrate for ribonucleoside diphosphate reductase.

To eliminate any contaminating ADP and to prevent the accumulation of ADP while a solution sat in the syringe, pyruvate kinase and PEP were mixed with the ATP. It was determined in a second set of control experiments that after 15 min in the syringe, even with this ATP-regenerating system present, cysteine 225 and cysteine 462 began slowly to form a cystine in samples containing R1 protein, R2 protein, ATP, pyruvate kinase, and PEP, but if ATP was omitted, less than 5% of the cystine between cysteine 225 and cysteine 462 had formed after 45 min. These results suggested that even with the pyruvate kinase and PEP, enough ADP was present to oxidize the enzyme slowly over time. To avoid this slow oxidation of the enzyme in the syringe, which became apparent only after 15 min, all time points were collected less than 5 min following the mixing of ATP, pyruvate kinase, and PEP with R1 and R2 proteins.

Once these controls had been run, a time course for the formation of the cystine between cysteine 225 and cysteine 462 could be measured in the quench flow apparatus. For each time point, a solution of ATP, pyruvate kinase, and PEP in HEPES buffer containing EDTA and $MgSO_4$ was added to a sample of R1 protein and R2 protein in the same buffer, and this mixture was immediately loaded into the sample syringe of the quench flow instrument. The other syringe contained CDP and HEPES buffer with EDTA and $MgSO_4$, and two runs were collected in 5 min for a particular time point. The concentrations of enzyme and nucleotides upon mixing the effluents from the two syringes were 3 μM R1 dimer, 15 μM R2 dimer, 3 mM ATP, 25 mM PEP, 0.04 mg mL^{-1} pyruvate kinase, 1 mM CDP, 1 mM EDTA, 15 mM $MgSO_4$, and 50 mM HEPES, pH 7.6. As time progressed, the percentage of cysteine 225 participating in the cystine increased (Figure 1).

An unidentified peak of fluorescence (peak a, Figure 1D) was evident in many of the chromatograms. A separate experiment was performed to generate a greater quantity of this component. R1 protein (10 nmol) was mixed with a 5-fold molar excess of R2 protein, as well as with CDP and HEPES buffer containing EDTA and $MgSO_4$, and this mixture reacted for 5 min at room temperature before it was manually quenched with a solution of acetic acid containing NEM. The protein was precipitated and digested with chymotrypsin and the proteinase from *S. aureus* strain V8, and the digest was passed over the immunoadsorbent. The immunoadsorbent was washed with PBS, and those peptides that were specifically bound were eluted with 0.5 M acetic acid. Acidic fractions were pooled, concentrated, and submitted to HPLC. A peak of absorbance at 229 nm was observed at 33 min. A small portion of the fraction associated with this peak was modified with the AQHC and submitted to chromatography on the system equipped to monitor fluorescence. The single peak of fluorescence that was observed on the chromatogram had the same retention time (35 min) as the unidentified peak (peak a, Figure 1D). Another portion of this fraction was sequenced. The amino acids observed on the chromatograms from the first seven cycles were S, S + T, L, V, L, I, and E. The amino acid composition following acid hydrolysis of another portion of the fraction associated with this peak was $S_{1.8}V_{1.2}L_{2.2}I_{1.1}E_{1.3}T_{1.0}C-C_{0.8}$. These results

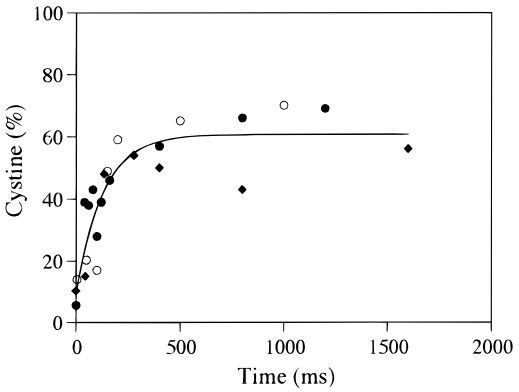

FIGURE 2: Percent cystine formed between cysteine 225 and cysteine 462 upon mixing with CDP as a function of time in the presence of ATP. Ribonucleoside diphosphate reductase was mixed in a quench flow apparatus with CDP at the same concentration of reactants and as described in Figure 1. The areas of 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 were measured for each time point from the chromatograms of effluents from the immunoadsorbent (Figure 1). These areas and the relative yields of fluorescence for each peptide were used to calculate the percent cystine at each time point. Percent cystine is presented as a function of time (milliseconds). Solid squares, open circles, and solid circles represent three separate experiments. The data were fit with an equation with a single-exponential term to obtain a first-order rate constant of 8 s^{-1}. From the fit of all of the points of the data, the amount of cystine at zero time was 9%, and the maximum yield of cystine was 61%.

demonstrate that the peak on the chromatograms of the experimental samples with the retention time of 35 min is the mixed disulfide between CTL and SSCVLIE resulting from the digestion of R1 protein, presumably by the chymotrypsin, between leucine 461 and cysteine 462. In a separate experiment, the yield of fluorescence for the fluorescent adduct of this mixed disulfide between SSCVLIE and CTL was determined and found to be the same as the yield of fluorescence for the fluorescent adduct of the mixed disulfide between SSCVLIE and IALCTL. Therefore, the areas of the fluorescence from both of these mixed disulfides were added together to calculate the percent cystine formed between cysteine 225 and cysteine 462.

The data from three experiments (Figure 2) in which R1 protein, R2 protein, and ATP were mixed with CDP were fit to an equation with a single-exponential term to obtain a first-order rate constant of 8 s^{-1}. The first-order rate constants calculated separately from each set of data were 11 s^{-1}, 7 s^{-1}, and 6 s^{-1}, so the range of the values for the rate constant was ± 3 s^{-1}. To ensure that no oxidation was occurring in the absence of CDP, controls in which CDP was omitted from the runs were performed in the quench flow apparatus. Two runs were collected within 5 min of the addition of ATP, pyruvate kinase, and PEP to the mixture of R1 and R2 proteins. The control samples were quenched 2 s after mixing and the percent cystine between cysteine 225 and cysteine 462 was calculated. For the experiment described in Figure 1, the percent cystine in these controls was found to be less than 5% (Figure 1C), demonstrating that nearly all the cystine between cysteine 225 and cysteine 462 was the result of mixing the enzyme with CDP. The values for these controls from all three of the experiments were used for the points at zero time in Figure 2. From the fit to all of

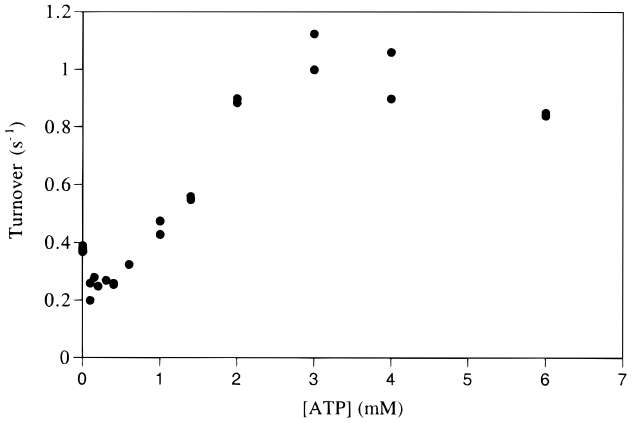

FIGURE 3: Steady-state turnover number as a function of the concentration of ATP. The coupled assay of Thelander et al. (*30*) was used to determine the steady-state activity of ribonucloside diphosphate reductase at various concentrations of ATP. The concentrations of other reagents were 3 μM R1 dimer, 15 μM R2 dimer, 40 μM thioredoxin, 1 μM thioredoxin reductase, 15 mM $MgSO_4$, 1 mM NADPH, 1 mM CDP, and 50 mM HEPES, pH 7.6. The turnover number was calculated by dividing the initial velocity observed in the assay by the molar concentration of R1 monomer present in the assay.

the points of the data, the amount of cystine at zero time was 9% and the amplitude of the exponential phase was 52% cystine.

Rate of Formation of Cystine in the Absence of ATP. The rate of reduction of CDP in the steady-state assay (Figure 3) is enhanced by the addition of ATP (*6*, *9*). In the steady-state assay, the turnover number of R1 protein at the concentrations used in the quench flow apparatus in the presence of ATP was 1.1 μmol of oxidized thioredoxin (μmol of active site)$^{-1}$ s^{-1}, while in the absence of ATP, it was 0.4 s^{-1}. To see if this enhancement produced by ATP could be detected in the single-turnover experiments, I repeated the experiments in the quench flow apparatus exactly as before except without ATP and the ATP regenerating system. The percent cystine formed between cysteine 225 and cysteine 462 at each time point was calculated, and the data from two separate experiments (Figure 4) were fit to an equation that was the sum of a single exponential and a line to obtain a rate constant of 1.5 s^{-1} for the exponential phase. The first order rate constants calculated separately from each set of data were 1.3 s^{-1} and 1.7 s^{-1}, so the range on the rate constant was $\pm$0.2 s^{-1}. Controls in which CDP was omitted and samples were quenched 10 s after mixing were performed after all the time points had been collected. The percent cystine between cysteine 225 and cysteine 462 was less than 4% in these controls. The values of the percent cystine for these controls from the two separate experiments were used for the points at zero time in Figure 4. From the fit to the data, the amount of cystine at zero time was 4% and the amplitude of the exponential phase was 37% cystine.

Rate of Formation of Cystine in the Presence of ATP at High Concentrations of Enzyme. One way to decrease the turnover number of ribonucleotide reductase is to remove ATP from the solution (Figure 3); another is to increase the concentration of the enzyme (Figure 5). At 15 μM R1 dimer and 75 μM R2 dimer (90 μM tyrosyl radical), the turnover number of the enzyme (0.04 s^{-1}) is only 4% of its value (1 s^{-1}) at 3 μM concentration of R1 dimer (18 μM of tyrosyl radical). This remarkable decrease in enzymatic activity is

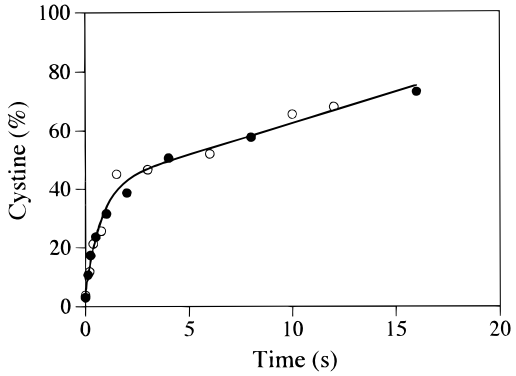

FIGURE 4: Percent cystine formed between cysteine 225 and cysteine 462 upon mixing with CDP as a function of time in the absence of ATP. Ribonucleoside diphosphate reductase in the absence of ATP, pyruvate kinase, and PEP was mixed in a quench flow apparatus with CDP at the same concentration of reactants and 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 were used to calculate the percent cystine at each time point. Percent cystine is presented as a function of time (seconds). Open and solid circles represent two separate experiments. The data were fit with an equation that was the sum of a single exponential and a line to obtain a first-order rate constant of 1.5 s^{-1} for the exponential phase and a zero-order rate constant of 2% cystine s^{-1} for the linear phase. From the fit to all the points of the data, the amount of cystine at zero time was 4%, and the amplitude of the exponential phase was 37% cystine.

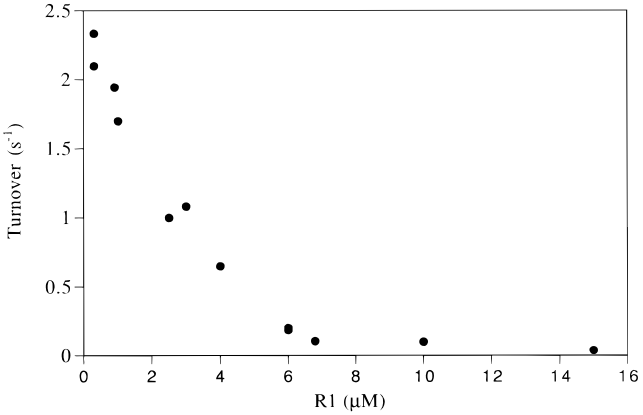

FIGURE 5: Effect of increasing the concentration of R1 protein on the steady-state turnover number. The coupled assay of Thelander et al. (*30*) was used to determine the steady-state activity of ribonucloside diphosphate reductase at various concentrations of R1 protein. A 5-fold molar excess of R2 protein over R1 protein was used in each assay. The concentrations of all other reagents were 40 μM thioredoxin, 1 μM thioredoxin reductase, 15 mM $MgSO_4$, 1.6 mM ATP, 1 mM NADPH, 1 mM CDP, and 50 mM HEPES, pH 7.6. The turnover number was calculated by dividing the initial velocity observed in the assay by the molar concentration of R1 monomer present in the assay.

not due to inadequate concentrations of either thioredoxin or thioredoxin reductase in the steady-state assays because doubling their concentrations had no effect on the results. It was of interest to examine the effect of increasing the concentration of protein on the rate of cystine formation. In these experiments at high concentrations of enzyme, R1 protein and R2 protein were added to separate syringes to prevent the oxidation of the enzyme by contaminating ADP. Without the R2 protein, R1 protein is catalytically inactive and, therefore, cannot be oxidized by ADP, but preparations

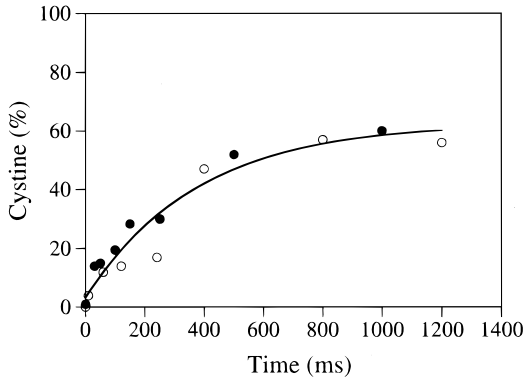

FIGURE 6: Percent cystine formed from cysteine 225 and cysteine 462 upon mixing with CDP as a function of time at a high concentration of enzyme. Prereduced R1 protein that had been treated with hydroxyurea was mixed with ATP and CDP in HEPES buffer containing EDTA and $MgSO_4$ and loaded into one syringe of the quench flow apparatus. To the other syringe was added R2 protein in HEPES buffer containing EDTA and $MgSO_4$. Upon the mixing of the effluents from the two syringes, the final concentrations were 15 μM R1 dimer, 30 μM R2 dimer, 1.6 mM ATP, 1 mM CDP, 1 mM EDTA, 15 mM $MgSO_4$, and 50 mM HEPES, pH 7.6. 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 were used to calculate the percent cystine at each time point. Percent cystine is presented as a function of time (milliseconds). Open and solid circles represent two separate experiments. The data were fit with an equation with a single-exponential term to obtain a first-order rate constant of 2.6 s^{-1}. From the fit to all of the points of the data, the amount of cystine at zero time was 3%, and the maximum yield of cystine was 63%.

of R1 protein have to be treated with hydroxyurea to destroy any contaminating R2 protein before they are prereduced.

The prereduced R1 protein that had been treated with hydroxyurea was added along with ATP, CDP, and HEPES buffer containing $MgSO_4$ and EDTA to one syringe of the quench flow apparatus. To the other syringe was added R2 protein with HEPES buffer containing $MgSO_4$ and EDTA. Upon the mixing of the effluents from the two syringes, the concentrations were 15 μM R1 dimer, 30 μM R2 dimer, 1.6 mM ATP, 1 mM CDP, 1 mM EDTA, 15 mM $MgSO_4$, and 50 mM HEPES, pH 7.6. A solution of acetic acid containing NEM was used to quench the reaction. Since the concentration of R1 protein was higher than that used in the previous experiments, only one sample was collected for each time point to save enzyme. The percent cystine formed between cysteine 225 and cysteine 462 was calculated for each time point as before. The data from two separate experiments (Figure 6) were fit to an equation with a single-exponential term to obtain a first-order rate constant of 2.6 s^{-1}. The first-order rate constants calculated separately from each set of data were 2.3 s^{-1} and 2.9 s^{-1}, so the range on the rate was $\pm$0.3 s^{-1}. Controls in which the R2 protein was not present were performed after all the time points had been collected. The amount of cystine formed between cysteine 225 and cysteine 462 after 2 s in these controls was less than 2%. The values for the percent cystine in these controls from the two separate experiments were used for the points at zero time in Figure 6. From the fit to the data, the amount of cystine at zero time was 3% and the amplitude of the exponential phase was 60%.

Because the experiments just described were performed at different molar ration of R1 dimer to R2 dimer than those reported in Figure 5, steady-state assays were run under conditions identical to those used in the quench flow experiments of Figure 6. At 15 μM R1 dimer, 30 μM R2 dimer, 1.6 μM ATP, and 1 μM CDP, the steady-state turnover number of the enzyme was 0.2 s^{-1}.

DISCUSSION

It has been proposed (*3*, *4*, *15*) that formation of a cystine between cysteine 225 and cysteine 462 in the active site of R1 protein of ribonucleoside diphosphate reductase from *E. coli* precedes the completion of the reaction producing the deoxyribonucleotide. In the experiments of Lin et al. (*19*), cysteine 225, cysteine 230, cysteine 462, cysteine 754, and cysteine 759 were all identified as candidates for participation in the disulfides that form when prereduced R1 protein is oxidized enzymatically by CDP (*1*). Although the pairs of cysteines that had combined to form the cystines could not be identified, the results suggested that the R1 protein contain two pairs of cysteines that participate as reductants in the enzymatic reaction.

The behavior of two mutants, C225SR1 and C462SR1, led to the proposal that cysteine 225 and cysteine 462 provide both the necessary hydrogen atom and the necessary electron directly to the substrate during its reduction and have together become a cystine by the completion of turnover. Incubation of either mutant with R2 protein and a normal substrate for the enzyme, [3′-^{3}H,U-^{14}C]UDP, resulted in irreversible inactivation of the enzyme. In the case of the C225SR1 mutant (*18*), the inactivation resulted from the self-cleavage of the enzyme, and in the case of the C462SR1 mutant (*22*), the inactivation resulted from the covalent modification of the enzyme. During both the two separate reactions producing the respective inactivations, ^3H_2O was released from both mutants, and a tritium kinetic isotope effect at the 3′ carbon was observed (*18*, *22*). The existence of this kinetic isotope effect suggested that both mutant forms of the enzyme were able to proceed through the normal mechanism beyond the abstraction of the hydrogen from the 3′ carbon of the ribonucleotide and were able to form the 3′-radical intermediate. It was proposed that it was the inability of the mutants to form the cystine and thereby provide either the hydrogen atom or the second electron in the proposed mechanism, respectively, that short circuited the turnover of the substrate and led to side reactions producing the respective alterations of the enzyme (*18*).

The crystallographic molecular model of the ribonucleotide reductase from T4 bacteriophage (*23*) indicates that, contrary to what has been proposed for the enzymes from class 1, a cystine cannot form in its active site because the enzyme from T4 bacteriophage does not contain a cysteine homologous to cysteine 462 in the enzyme from *E. coli*. The current study, however, indicates that cysteine 225 and cysteine 462 do form a cystine during the turnover of the enzymes from class 1.

The first set of experiments on the quench flow apparatus (Figure 2) were performed at concentrations of R1 protein and ATP near the reported in vivo concentrations. The concentration of ATP in *E. coli* has been calculated to be 2.7 mM (*34*).[2] The concentration of R1 protein in *E. coli* was determined by rocket immunoelectrophoresis to be 2 μM (*35*). The concentrations of ATP and R1 protein used

Table 1: Kinetic Constants Observed at Different Concentrations of R1 Protein and ATP

[R1 dimer] (μM)	[R2 dimer] (μM)	[ATP] (mM)	calculated turnover[a] (s^{-1})	observed turnover (s^{-1})
3	15	3	4 ± 1.5	1.1
3	15	0	0.6 ± 0.1	0.4
15	30	1.6	1.5 ± 0.2	0.2

[a] The first-order rate constant for the formation of the cystine between cysteine 225 and cysteine 462 was multiplied by the fraction of active sites participating in the burst of cystine formation.

in the quench flow experiments were 3 mM and 3 μM, respectively.

The first-order rate constant for the formation of the cystine between cysteine 225 and cysteine 462 observed in these experiments was 8 ± 3 s^{-1} (Figure 2). The turnover number, when measured in the steady-state assay (Figure 3) at the same concentrations of R1 protein, R2 protein, pyruvate kinase, PEP, ATP, and CDP, was 1.1 s^{-1}. Because only 50% of the active sites of R1 protein participated in the burst of cystine formation observed in the quench flow experiments (Figure 2), it must be assumed that at any given instant only 50% of the active sites in the steady-state assay are participating in turnover. This assumption must be made because it increases the stringency of the test for kinetic competence. Applying this assumption, the maximum steady-state turnover number for the entire population of active sites, competent and incompetent, calculated from the first-order rate constant for the formation of cystine between cysteine 225 and cysteine 462 was 4 ± 1.5 s^{-1}. This maximum steady-state turnover number is greater than the observed turnover number of 1 s^{-1} in the steady-state assays (Table 1). Therefore, not only does the cystine between cysteine 225 and cysteine 462 form upon addition of CDP to the enzyme, but its formation is kinetically competent.

A maximum steady-state turnover for the enzyme in the absence of ATP can be calculated from the first-order rate constant for the formation of the cystine between cysteine 225 and cysteine 462 in the absence of ATP (Figure 4). The first-order rate constant for the formation of the cystine between cysteine 225 and cysteine 462 in the absence of ATP was 1.5 ± 0.2 s^{-1}. This number, when adjusted to account for the percentage of competent active sites (37%), yields a maximum steady-state turnover number of 0.6 ± 0.1 s^{-1}. The observed steady-state turnover number for R1 protein, under the same conditions in a steady-state assay (Figure 3), was 0.4 s^{-1}. This result suggests that when ATP is removed from the solution, the formation of the cystine between cysteine 225 and cysteine 462 becomes the rate-limiting step in the reaction.

Increasing the concentration of enzyme in the steady-state assays resulted in a significant decrease in the turnover number (Figure 5). Additional experiments were conducted to study what effect increasing the concentration of enzyme had on the rate for the formation of the cystine between cysteine 225 and cysteine 462. A different method, one that has been used in the laboratory of Dr. JoAnne Stubbe,[3] was employed to prevent the oxidation of R1 protein resulting from contaminating ADP. R1 protein and substrates were added to one syringe and R2 protein was added to the other syringe. The first-order rate constant for the formation of a cystine between cysteine 225 and cysteine 462 observed in these experiments performed at 15 μM R1 dimer was 2.6 ± 0.3 s^{-1} (Figure 6), while the steady-state turnover number of the enzyme at 15 μM R1 dimer and 30 μM R2 dimer was only 0.2 s^{-1}. Therefore, increasing the concentration of R1 protein from 3 to 15 μM resulted in a 5-fold reduction in the observed steady-state turnover number but only a 3-fold reduction in the first-order rate constant for the formation of cystine. The results demonstrate that, at the high concentration of enzyme, some step other than the formation of the cystine between cysteine 225 and cysteine 462 is rate-limiting.

In each of the quench flow experiments, a significant portion of the active sites of R1 protein did not participate in the burst of cystine formation. There are several possible explanations for the less than stoichiometric formation of the cystine in these experiments. First, R2 dimer as it is isolated (*3, 36*) always has equal to or less than 1.2 mol of radical for every mole of dimer. Even if every active site on every R1 monomer is properly connected to an R2 monomer, equal to or less than 60% of the R1 monomers should be connected to an R2 subunit containing a radical. Second, because it has never been observed and because there is no experimental evidence consistent with its existence, the rotationally symmetric, equimolar complex between an R1 dimer and an R2 dimer (*6*) that is usually assumed may never form. It is possible that, because of steric hindrance in whatever complex does form, at most only one of the subunits within an R1 dimer can be coupled to an R2 monomer in an R2 dimer. Third, if a complex of unexpected, higher stoichiometry forms among one R1 dimer and more than one R2 dimer, or more than one R1 dimer and one R2 dimer, or more than one R1 dimer and more than one R2 dimer, the structure of the complex could be such that only a portion of the R1 monomers within it would be properly associated with R2 monomers. It is possible that the slow increase in the concentration of cystine following the initial burst (Figure 4) reflects a slow rearrangement of R1 and R2 dimers during which initially unserviced active sites become effectively coupled to R2 monomers containing tyrosyl radicals.

[2] Because the in vivo concentration of ATP never varies, the effect of ATP on the enzyme has no regulatory significance.

[3] Personal communication.

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