Identification of an Active Site Residue of the R1 Subunit of Ribonucleotide Reductase from *Escherichia coli*: Characterization of Substrate-Induced Polypeptide Cleavage by C225SR1†

Wilfred A. van der Donk,‡ Chenhui Zeng,‡ Klaus Biemann,*,‡ and JoAnne Stubbe*,‡,§

Department of Chemistry and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

AnneMarie Hanlon and Jack Kyte*

Department of Chemistry, University of California at San Diego, La Jolla, California 92093

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ABSTRACT: Incubation of the C225S mutant of the R1 subunit of ribonucleotide reductase from *Escherichia coli* with the R2 subunit and nucleoside diphosphates leads to fragmentation of the polypeptide backbone of R1 [Mao, S. S., Holler, T. P., Bollinger, J. M., Jr., Yu, G. X., Johnston, M. I., & Stubbe, J. (1992) *Biochemistry 31,* 9744−9751]. The 26 and 60 kDa cleavage fragments were purified to homogeneity. The 26 kDa polypeptide was digested with Lys-C, and the peptides were partially purified by RP-HPLC. Mass spectrometric analysis (MALDI-TOF) of the HPLC fractions allowed the identification of the C-terminal peptide. The molecular mass of this peptide (2176) revealed that serine-224 constitutes its C-terminus, and further analysis of the distribution of its monoisotopic masses by FAB-MS indicated that Ser224 possesses a carboxamide rather than a carboxylate group. Treatment of the 60 kDa cleavage fragment with cyanogen bromide and subsequent MALDI-TOF analysis of the partially RP-HPLC purified peptides yielded a fraction containing its N-terminal peptide. This peptide was digested with trypsin, and the digestion mixture was purified by HPLC. Analysis of the fractions by MALDI-TOF identified the N-terminal peptide and determined a mass of 2222. This mass suggested valine 226 was the N-terminal residue (modified by an adduct of 28 mass units). Larger amounts of the C-terminal tetrapeptide of the 60 kDa fragment ($V_{226}LIE_{229}$) were obtained by complete digestion of the crude reaction mixture with endoproteinase Glu-C. The peptide mixture was then purified on an immunoadsorbent column containing immobilized antibodies raised against a synthetic peptide with the sequence KVLIE. After elution of the affinity-bound peptide, it was analyzed by CID-MS verifying that an adduct of 28 mass units was attached to valine 226. These results indicated that the amino group of Val226 is formylated. The localization of the residues at the cleavage site of C225SR1 provides a biochemical identification of the active site region of the R1 subunit of RDPR from *E. coli.* The details of the mechanism of cleavage remain to be elucidated.

Ribonucleotide reductases catalyze the rate-determining step in DNA biosynthesis, conversion of nucleotides into deoxynucleotides (Eriksson & Sjöberg, 1989; Stubbe, 1990b; Reichard, 1993b). The *Escherichia coli* RNR[1] is composed of two homodimeric subunits R1 and R2. R1 is the business end of RNR containing the binding site for purine and pyrimidine nucleotide substrates, the binding site for allosteric regulators that govern specificity and turnover number, and the five cysteines required for catalysis (Thelander, 1974; Lin et al., 1987; Åberg et al., 1989, Mao et al., 1992a−c). The reducing equivalents to produce dNDP are provided by oxidation of two of these cysteines within the active site. R2 contains the dinuclear iron center and tyrosyl radical cofactor essential for nucleotide reduction (Sjöberg et al., 1977; Reichard & Ehrenberg, 1983) and is thought to be responsible for generation of a thiyl radical on R1 via a series of coupled electron and proton transfer reactions (Stubbe, 1990a; Mao et al., 1992b; Uhlin & Eklund, 1994).

Substrate analogs (mechanism based inhibitors) (Thelander et al., 1976; Stubbe & Kozarich, 1980a,b; Sjöberg et al., 1983; Ator et al., 1984; Harris et al., 1984, 1987; Ator & Stubbe, 1985; Salowe et al., 1987, 1993) and protein analogs (site directed mutants) (Åberg et al., 1989; Mao et al., 1989, 1992a−c) have been used as probes to understand the mechanism of nucleotide reduction and to identify the

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* Corresponding authors. Telephone: (617) 253-1814. Fax: (617) 258-7500. E-mail: wilfred@stubbe-lab.mit.edu.

‡ Department of Chemistry.

§ Department of Biology.

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; t-BOC, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; CDP, cytidine 5′-diphosphate; α-CHCA, α-cyano-4-hydroxyl cinnamic acid; CID, collision-induced dissociation; dNTP, 2′-deoxynucleoside triphosphate; DTT dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid; FAB, fast atom bombardment; Gn·HCl, guanidine hydrochloride; Hse, homoserine; Hepes, *N*-(2-hydroxethyl)piperazine-*N*′-(2-ethanesulfonic acid); Lys-C, endoproteinase Lys-C; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PBS, phosphate buffered saline; PFL, pyruvate formate lyase; PP_i, inorganic pyrophosphate; RNR, ribonucleotide reductase; SA, sinapinic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK, tosylphenylalanine chloroketone; Tris, tri-(hydroxymethyl) aminomethane; Y·, tyrosyl radical on the R2 subunit.

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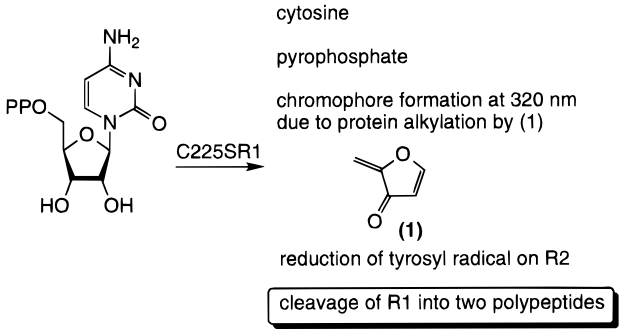
Scheme 1



functional groups involved in catalysis. The latter studies in conjunction with the biochemical investigations of Thelander (1974) and Lin et al. (1987) have established the requirement for five cysteines in catalysis. These studies elicited the proposal that Cys225 and Cys462 are the cysteines that directly provide the reducing equivalents to generate dNDPs, C439 is the thiyl radical that initiates nucleotide reduction by 3′-hydrogen atom abstraction from the NDP substrate, and Cys754 and Cys759 transfer reducing equivalents from the in vivo reductant, thioredoxin, into the active site by disulfide interchange (Stubbe, 1990a; Mao et al., 1992b). These proposed functions are supported by the recent crystal structure of R1 in which Cys439, Cys225, and Cys462 are within 6 Å of one another and Cys225 and Cys462 are present in a disulfide linkage (Uhlin & Eklund, 1994).

Despite the large number of potent and specific mechanism-based inhibitors of RNR, none have labeled an active site residue(s) with sufficient stability to allow its (their) isolation and identification. Recent studies with a site-directed mutant of R1, C225S, in which the reducing equivalents for the normal reduction process are not readily available, have provided the first chance to identify an active site residue on R1 (Mao et al., 1989, 1992a). Incubation of this mutant with cytidine 5′-diphosphate (CDP) and allosteric effector turns the normal substrate into a mechanism-based inhibitor. Detailed studies of this system have revealed that inactivation is initiated by 3′-C−H cleavage which is subsequently accompanied by formation of cytosine, inorganic pyrophosphate (PP_i), and 2-methylene-3(2*H*)-furanone (Scheme 1) (Mao et al., 1992a). Moreover, the inactivation results from loss of the tyrosyl radical on R2, which is accompanied by stoichiometric fragmentation of the R1 polypeptide backbone into two polypeptides of ≈60 and ≈ 26 kDa. The detailed studies on this R1 mutant have provided the first direct evidence that tyrosyl radical reduction on the R2 subunit is correlated with substrate 3′-carbon−hydrogen bond cleavage, as an isotope effect on tyrosyl radical loss is observed in the presence of [3′-^{2}H]UDP (Mao et al., 1992a). Studies in the present paper provide direct evidence for location of the enzyme's active site by defining both the site of cleavage of the polypeptide backbone of R1 and the nature of the fragmentation products using mass spectrometry.

MATERIALS AND METHODS

Materials. R1-C225S (ϵ_{280} 189 000 M^{-1} cm^{-1}) was isolated from the overproducing strain K38/C225SR1 as previously described (Mao et al., 1989), and R2 (specific activity 7700∼8000 nmol min^{-1} mg^{-1}, $\epsilon_{280} = 130\,500$ M^{-1} cm^{-1}) was prepared as described by Salowe & Stubbe (1986). *E. coli* thioredoxin (TR) was isolated from overproducing strains SK3981 with a specific activity of 36 units mg^{-1} (Lunn et al., 1984), and thioredoxin reductase (TRR) was isolated from K91/pMR14 with a specific activity of 1000 units mg^{-1} (Russel & Model, 1985). Adenosine 5′-triphosphate (ATP), CDP, iodoacetamide, TPCK-treated trypsin, catalase, and Sepharose CL 6B-200 gel filtration resin were obtained from Sigma. Trifluoroacetic acid (TFA) and cyanogen bromide was purchased from Aldrich, and Lys-C and glucose oxidase were obtained from Boehringer Mannheim. Endoproteinase Glu-C from *Staphylococcus aureus* was obtained from Worthington, and t-BOC amino acids were from CalBiochem. Dithiothreitol (DTT) was purchased from Malinckrodt. Urea (molecular biology grade) was obtained from American Bioanalytical and was de-ionized by passing through a mixed-bed Dowex-AG-501-X8 (D) resin from Bio-Rad. Centricon microconcentrators and ultrafiltration membranes were obtained from Amicon Co.

General Methods. A PerSeptive Voyager Elite or a VT2000 time-of-flight mass spectrometer equipped with a N_2 laser (337 nm) and using +30 kV accelerating voltage was used for MALDI-TOF (matrix assisted laser desorption ionization-time-of-flight) measurements. FAB-MS was carried out in the first (MS-1) of two mass spectrometers of a tandem high-resolution JEOL HX110/HX110 mass spectrometer at an accelerating voltage of 10 kV, a collision energy of 7 kV, and a mass resolution of 1:2000 (10% valley). Spectra were acquired and processed using a JEOL Complement system. $(CsI)_nCs^+$ cluster ions were used for calibration. The primary Cs^+ ions were produced in a JEOL Cs^+-ion gun operated at 26 kV.

HPLC was performed on a Beckman System Gold with a 0.46 × 25 cm Vydac C-18 RP-column. Solvent system I consisted of solvent A (0.1% aqueous TFA) and solvent B (80% aqueous acetonitrile, 0.07% TFA). Solvent system II consisted of solvent C (5 mM potassium phosphate buffer, pH 6.2) and solvent D (80% acetonitrile, 20% solvent C). HPLC purification of a CNBr digest was carried out on a Waters HPLC system with a 0.46 × 25 cm Vydac C-4 RP-column using solvent system III consisting of buffer E (0.05% TFA in H_2O) and buffer F (0.035% TFA in acetonitrile).

Acid hydrolyses of peptides were performed in 6 M hydrochloric acid under argon at 160 °C for 45 min, and the resulting hydrolysates were analyzed using a modular system composed of a Spectra-Physics autosampler SP8875 Spectra-Physics ternary pump SP8800 and a Pickering sodium cation-exchange analytical column with a postcolumn derivatization of amino acids with ninhydrin. Laser densitometry was performed using a LKB Bromma 2202 Ultroscan.

Large Scale Cleavage Reaction. A typical reaction mixture contained in a final volume of 10 mL Hepes assay buffer (50 mM Hepes, 15 mM $MgSO_4$, 1 mM EDTA, pH 7.6), 20 μM C225SR1, 60 μM R2, 1.6 mM ATP, and 1.6 mM CDP. Prior to the addition of protein the mixture was purged with O_2 for 30 min. The incubation was carried out at 25 °C for 2 h in a reaction vessel open to the atmosphere. The resulting solution was used directly for isolation of the cleavage products.

Purification of the 26 and 60 kDa Fragments. The crude reaction mixture was loaded onto a 2.5 × 40 cm dATP Sepharose affinity column (Berglund & Eckstein, 1974) and eluted with 50 mM Tris and 10 mM DTT, pH 7.6. After elution of R2 and the small molecular weight reactants (∼250 mL), the column was washed with an additional 200 mL of buffer. The eluent was then changed to 50 mM Tris, 10 mM DTT, and 10 mM ATP, pH 7.6, and the first 50 mL of flow through containing ATP was pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. The ATP concentration was reduced below 10 μM by consecutive dilutions with 50 mM Tris and 10 mM DTT, pH 7.6, and subsequent reconcentrations. All procedures were carried out at 4 °C.

The resulting solution contained intact R1, the 26 kDa fragment and the 60 kDa fragment as monitored by 10% polyacrylamide SDS−PAGE (Laemmli, 1970). The solution was further concentrated by centrifugation using a Centricon-3 (Amicon Co.), and the buffer was exchanged, at 25 °C, to 50 mM potassium phosphate and 1.8 mM sodium dodecyl sulfate (SDS), pH 7.0. The protein solution was loaded onto a Sepharose CL 6B-200 column (1.5 × 75 cm) equilibrated in 50 mM potassium phosphate, 1.8 mM SDS, and 0.02% NaN_3 and eluted at a flow rate of 4.5 mL h^{-1}. Fractions (1 mL) were collected and analyzed by UV spectroscopy (A_{280}) and SDS−PAGE (10% acrylamide) showing partial separation of the 26 kDa fragment from the two larger polypeptides. The protein fractions enriched in the 26 kDa fragment were concentrated and reloaded onto the Sepharose column. The elution procedure was repeated yielding a solution of homogeneous 26 kDa fragment.

The 60 kDa fragment was isolated via a different procedure. After removal of R2 from the crude cleavage reaction mixture by dATP affinity chromatography (see above), the resulting protein solution was loaded onto a 7.5% SDS−PAGE (3 mm) protein gel divided into 10 slots. After running the gel a small slice was removed and stained with Coomassie Brilliant Blue. The band corresponding to the 60 kDa fragment was isolated from the remaining unstained gel and cut into small gel slices which were electroeluted for 3 h at 280 V using an Amicon microelectroeluter in combination with Centricon-30 microconcentrators. The elution buffer consisted of 12.5 mM Tris, 100 mM glycine, and 1.25% SDS. The overall yield for the isolation of the 60 kDa fragment using this procedure was typically 25−30%.

Lys-C Digestion, Peptide Mapping, and MS analysis of the 26 kDa Fragment. The 26 kDa fragment was exchanged into 50 mM Tris-acetate and 6 M deionized urea, pH 7.8, and residual SDS was removed from the protein by repeated dilutions and reconcentrations by ultrafiltration with an Amicon PM-10 membrane. Prior to the addition of Lys-C, the urea concentration was reduced to 2 M by dilution with 50 mM Tris-acetate buffer (pH 7.8). A Lys-C solution prepared in water directly before use (∼1 mg mL^{-1}) was added to the protein solution to give a final ratio of 1:15 (w/w) Lys-C/26 kDa protein. The digest was incubated at 37 °C for 16 h. A control digest containing only Lys-C was performed simultaneously.

The digest was chromatographed using solvent system I (see *General Methods*) in two fractions at a flow rate of 0.8 mL min^{-1}. After injection the mobile phase was changed linearly over 60 min from 98% solvent A/2% solvent B to 63% solvent A/37% solvent B. Over the following 30 min the percentage of solvent B was further increased to 75% and finally to 98% over an additional 15 min. Fractions were collected every minute in polypropylene microcentrifuge tubes. The solvent was then removed using a speed-vac, and the residues in the individual tubes were analyzed by MALDI-TOF mass spectrometry. The residues from the lyophilized HPLC fractions of the Lys-C digest were redissolved in 30% acetonitrile and mixed with the matrix (α-cyano-4-hydroxylcinnamic acid, α-CHCA) to a final concentration of 1−3 pmol/μL, and 0.5 μL of this solution was used to obtain the MALDI spectra. The mass measurements were carried out using external calibration providing an accuracy of ±0.1%.

After identification of a candidate peptide in one of the fractions, the fraction containing this peptide was further fractionated to give homogeneous peptide using two different methods. The first method used solvent system II with a linear gradient from 98% solvent C/2% solvent D to 60% solvent D over 60 min, followed by a linear gradient from 60% solvent D to 98% solvent D over 30 min. The flow rate was 0.8 mL min^{-1}. The second purification involved chromatography using solvent system I with a linear gradient from 90% solvent A/10% solvent B to 60% solvent B over 150 min. Sequence analysis of this purified peptide was performed by automated Edman degradation in the MIT biopolymers laboratory.

For FAB-MS measurements the peptide was dissolved in about 50% glycerol and thioglycerol to give a final concentration of 0.4−1.0 nmol/μL, and 0.5−1.0 μL of this solution was loaded to the target for spectra acquisition.

Cyanogen Bromide (CNBr) Digestion and Peptide Mapping of the 60 kDa Fragment. The electroeluted 60 kDa fragment sample (6 nmol) was diluted with 2 M deionized urea and 0.1 M NH_4HCO_3, pH 7.8 and reconcentrated in a Centricon-30 microconcentrator. After five repetitions of this procedure to remove residual SDS, the resulting solution (280 μL) was treated with 20 μL of 110 mM DTT, and the sample was incubated for 1 h at 37 °C. The solution was cooled to 25 °C, 50 μL of a 100 mM solution of iodoacetamide was added (90 equiv per cysteine residue), and the mixture was incubated for 1 h and 30 min at 25 °C in the dark. Then 40 mg of solid trichloroacetic acid (TCA) was added to a final concentration of 10%, and the suspension was cooled at 0 °C for 40 min. The formed suspension was then pelleted using a microcentrifuge, and the supernatant was removed. The protein pellet was washed two times with cold acetone and then redissolved in 50 μL of 70% aqueous trifluoroacetic acid (TFA). A CNBr solution was prepared by dissolving 500 mg in 1.5 mL of acetonitrile and 20 μL (6 μmol, 100 equiv per methionine) of this solution was added to the protein sample in an Eppendorf tube flushed with argon. The reaction mixture was stored in the dark for 24 h at 25 °C. The solution was then diluted with 1 mL of water and lyophilized to dryness. The crude digest was fractionated on a Vydac C-4 column (0.46 × 25 cm) with a gradient of 95% solvent E/5% solvent F (see *General Methods*) to 35% E/65% F in 90 min at a flow rate of 0.8 mL min^{-1}. After the HPLC fractions were screened by MALDI-TOF MS, the fraction containing the N-terminal peptide was rechromatographed using a shallower gradient of 70% E/30 % F to 55% E/45% F in 60 min at a flow rate of 0.5 mL min^{-1}. Tryptic digestion of the candidate peptide was carried out in a

PerSeptive trypsin column (0.5 × 3 cm, Poroszyme). The column was eluted with buffer containing 0.05 M Tris-HCl (pH 8.2) and 0.05 M $CaCl_2$. The total digestion time in the column was 30 min at a flow rate of 0.01 mL min^{-1}. The tryptic peptides were collected in a C-18 guard column and washed with solvent E until the pH of the eluate was 2.5, and a Vydac C-18 column (0.46 × 25 cm) was then connected. The column was eluted using a linear gradient of 95% A/5% B to 45% A/55% B in 50 min. Fractions containing individual peptides were collected and analyzed by MALDI-MS (see above).

Peptide Synthesis. The peptide with the sequence KVLIE was synthesized by conventional solid-phase methods for t-BOC amino acids (Stewart & Young, 1984). The first amino acid, N^{α}-t-BOC-L-glutamic acid O^{γ}-benzyl ester, was attached to chloromethyl resin with potassium fluoride. The following L-amino acids were added in order: N^{α}-t-BOC-L-isoleucine, N^{α}-t-BOC-leucine, N^{α}-t-BOC-L-valine, and N^{α}-t-BOC-N^{ϵ}-(2-chlorobenzyloxycarbonyl)-L-lysine. Each amino acid was added to the chain as a preformed hydroxybenzotriazole ester. The product was cleaved by bubbling anhydrous hydrobromic acid through the resin in 95:5 TFA/anisole, and the TFA was removed under reduced pressure. The residue remaining after removal of the TFA was dissolved in 10% methanol and extracted with diethyl ether. The aqueous phase was lyophilized, and the resulting crude product was purified by reverse-phase HPLC. Following acid hydrolysis, the amino acid composition of the peptide was found to be $K_{1.0}V_{0.9}L_{1.1}I_{1.2}E_{1.0}$. Leucine aminopeptidase and carboxypeptidase Y digestions confirmed the composition of the peptide yielding the compositions $K_{1.1}V_{1.0}L_{1.1}I_{0.9}E_{1.0}$ and $K_{0.1}V_{0.3}L_{0.9}I_{1.0}E_{1.1}$, respectively.

Preparation of Immunoadsorbents. A haptenic conjugate of the peptide KVLIE and bovine serum albumin (BSA) was used to produce polyclonal antipeptide immunoglobins in New Zealand White rabbits. The peptide was coupled to the BSA through its amino terminus with the cross-linking reagent glutaraldehyde (Kagen & Glick, 1979). BSA (10 mg in 0.5 mL) and peptide (2 μmol in 2 mL) were dissolved in 0.4 M sodium phosphate, pH 7.5, and 3% glutaraldehyde (1 mL) was added dropwise over 5 min at room temperature. The reaction continued for 30 min at room temperature before it was quenched with 1 M glycine (250 μL) for an additional 30 min. The product was dialyzed against phosphate buffered saline (PBS) and hydrolyzed with acid to determine the extent of coupling. The ratio for the conjugate was 9 nmol of peptide to every nanomole of BSA. The conjugate (0.3 mg mL^{-1}) as an emulsion in Freund's complete adjuvant was injected intramuscularly for the initial immunization. Subsequent injections were made with an emulsion of conjugate and Freund's incomplete adjuvant.

An immunoadsorbent for immunoglobins recognizing the sequence -VLIE was constructed by coupling the peptide KVLIE to a solid support. A solution of peptide (10 μmol in 1 mL) was added to 1 mL of Affi-Gel 10 in PBS, and the solution was agitated at room temperature overnight. Uncoupled peptide was removed by washing the resin.

Antiserum was passed over the immunoadsorbent, the resin was washed with PBS, and the desired, bound antibodies were eluted with 0.1 M sodium phosphate, pH 2.5, and dialyzed into PBS. The affinity purified immunoglobins were concentrated in a Centricon 30 centrifugal concentrator (Amicon) to a final volume of 1 mL and coupled to Affi-Gel 10 as described above at 4 °C. This immunoadsorbent was then poured into a column. The capacity of this immunoadsorbent to bind KVLIE was determined by saturating the column with synthetic peptide, washing the column with PBS and eluting bound peptide with 0.1 M sodium phosphate, pH 2.5. The acid eluates were submitted to HPLC, and the peptide was quantified by amino acid analysis following acid hydrolysis. This immunoadsorbent had a binding capacity of 4 nmol of peptide per mL of resin.

Determination of the Cleavage Site by Immunoadsorbent Chromatography. Purified 60 kDa fragment (10 nmol in 4 mL) was digested with 100 μg of endoproteinase Glu-C from *S. aureus* in 50 mM Tris-HCl, 5 mM $Mg(OAc)_2$, and 4 mM DTT, pH 7.6, for 4 h at 37 °C. The resulting digest was the diluted with PBS and passed over the immunoadsorbent. Nonspecifically bound peptides were removed by a wash with PBS, and bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. The acid eluates were submitted to HPLC on a Vydac RP-18 column (1 cm × 28 cm). The column was eluted at 1 mL min^{-1} with a mobile phase gradient which was developed linearly over 40 min from 0.05% TFA in water to 0.038% TFA in 60/40 water/acetonitrile. The effluent from the column was continuously monitored ($A_{229\ nm}$) using a Waters HPLC Chromatograph with a fixed wavelength Waters UV−vis detector. Acid eluates contained 3 nmol of peptide which eluted at 29 min with a composition $V_{1.3}L_{1.0}I_{1.1}E_{0.9}$ determined by acid hydrolysis.

Cleavage of C225SR1 in ^2H_2O and Determination of the Fragmentation Site. Hepes assay buffer was prepared in ^2H_2O by lyophilization of 10 mL of a stock solution in H_2O, redissolving the residue in an equal volume of ^2H_2O, and adjusting the pD to 7.6 with 1 M HCl in ^2H_2O. Stock solutions of C225SR1 and R2 in Hepes assay buffer (^2H_2O) were prepared by repeatedly (3×) concentrating the protein samples by centrifugation using a Centricon-30 and redilution with Hepes assay buffer in ^2H_2O. Stock solutions of CDP, DTT, and ATP were prepared by dissolving the reagents in Hepes assay buffer in ^2H_2O. The solution of C225SR1 in deuterated assay buffer was prereduced with 20 mM DTT (^2H_2O) for 10 min and then immediately used. The cleavage reaction contained in a final volume of 6 mL of 50 mM Hepes assay buffer: 9 μM C225SR1, 27 μM R2, 1.3 mM ATP, 0.8 mM CDP, and 1.3 mM DTT. The work-up and the isolation of the *N*-terminal peptide of the 60 kDa fragment by the immunoadsorbent chromatography were identical to that described above. This peptide was analyzed by FAB-MS and CID-MS.

Cleavage under Anaerobic Reaction Conditions. Two different methods were employed to achieve anaerobic conditions.

Method A. The reaction mixture contained in a final volume of 400 μL degassed Hepes or Tris assay buffer: 16 μM C225SR1, 47 μM R2, 1.6 mM ATP, 1.0 mM CDP, 10 mM DTT, and 0.1 mM $Fe(NH_4)_2(SO_4)_2$. The proteins, ATP, DTT, and $Fe(NH_4)_2(SO_4)_2$ were mixed under an argon atmosphere in a cuvette equipped with a septum, and the solution was monitored by UV−vis spectroscopy. No loss of the tyrosyl radical signal at 412 nm was observed, and a control sample showed no loss of activity under these conditions. After 15 min CDP was added, the reaction was monitored at 412 nm, and the amount of radical lost was quantitated by the method of Bollinger et al. (1991). Two

control reactions were performed in which either CDP or $Fe(NH_4)_2(SO_4)_2$ was omitted. After 30 min, 5 μL aliquots were removed from the reaction mixtures, boiled in Laemmli buffer, and analyzed by 10% acrylamide SDS−PAGE. After the gel was stained with Coomassie Brilliant Blue, the individual lanes were scanned with a laser densitometer.

Method B. Aliquots (100 μL) of a stock solution containing 0.015 mM C225SR1, 0.015 mM R2, 1.6 mM ATP, 15 mM $MgSO_4$, and 100 mM glucose in 50 mM Hepes or Tris buffer (pH 7.5) were added to three septa sealed reaction vessels that were subsequently purged with argon. A fourth reaction vessel contained a solution of 16 mM CDP and 100 mM glucose in the same buffer. All solutions were degassed by three freeze/pump/thaw cycles, and aliquots of a stock solution of glucose oxidase and catalase were added under an atmosphere of argon to the first reaction vessel (reaction A) and to the CDP containing vessel giving final concentrations of 500 units/mL glucose oxidase and 750 units/mL catalase. The two remaining reaction mixtures (reactions B and C) served as controls. After incubation for 20 min at 25 °C, the septum was removed from the vessel of reaction C for an aerobic control. Aliquots of the CDP solution were then added to all three reaction mixtures to a final concentration of 1.0 mM. The reactions were left at 25 °C for 2 h and were then analyzed as described in method A.

RESULTS

Our previous studies revealed that incubation of CDP with C225SR1 and R2 resulted in conversion of the normal substrate into a mechanism-based inhibitor, loss of the tyrosyl radical on the R2 subunit, and cleavage of the R1 subunit into two polypeptides. In order to define the site of cleavage and the fragmentation products, conditions were varied to maximize the cleavage event and thus facilitate the goal at hand.

Large Scale Cleavage Reaction. During our studies on C225SR1 it had been observed that reactions carried out at low protein concentrations (2−10 μM) generally gave cleavage to a larger extent than reactions performed at higher concentrations. These observations suggested that molecular oxygen could be essential for this fragmentation and that at higher protein concentrations the oxygen content of the buffer became limiting. Optimal cleavage was indeed observed in the present studies for reactions carried out in buffer that was presaturated with oxygen (data not shown). In addition, given that there are only ∼1.2 tyrosyl radicals per R2, the maximum amount of cleavage of R1 (a homodimer), since it is directly correlated with tyrosyl radical loss, would be 0.6 equiv. Therefore, the ratio of R1:R2 was varied (1:1 to 1:3) in an effort to increase fragmentation. The present studies show that cleavage can be obtained reproducibly at moderate protein concentrations (20−50 μM) using O_2-saturated solutions and a 3-fold excess of R2 over R1. Large scale reactions were performed with open reaction vessels and with continuous slow stirring. Even under these optimized conditions, however, cleavage was limited to ∼50−60% of all R1 polypeptide chains.

Purification of the 26 and 60 kDa Fragments. In order to define the site of fragmentation, it was necessary to develop a method to separate the 26 and 60 kDa protein fragments from each other and from uncleaved R1 and R2. As a first step in the purification of the cleavage products, the R2 subunit was separated from R1 and the 26 and 60 kDa fragments using dATP affinity chromatography (Berglund & Eckstein, 1974).[2] Both the 26 and 60 kDa fragments eluted with intact R1 when the column was washed with buffer containing 10 mM ATP. These results indicate that R1 is present in a trimeric form after fission of one of its polypeptides, that is, the 26 and 60 kDa fragments stay associated with the 86 kDa protomer. Gel filtration chromatography on Sephacryl S-200 also indicated that R1, the 26 kDa fragment, and the 60 kDa fragment co-eluted. Efforts were then focused on separation of the three polypeptides. Initially, gel filtration chromatography on Sephadex G-100 or Sephacryl S-200 in the presence of 2 M urea was carried out without success. However, purification of the 26 kDa fragment was achieved by chromatographing the mixture on two successive Sepharose CL 6B columns eluting with a 50 mM potassium phosphate buffer (pH 7.0) containing 1.8 mM SDS. Although the 26 kDa fragment was obtained in homogeneous form as judged by SDS−PAGE, the 60 and 86 kDa polypeptides were not resolved by this method. Separation of the 60 kDa fragment from intact R1 by several gel filtration methods under a variety of denaturing conditions also proved unsuccessful. Homogeneous 60 kDa fragment was therefore obtained by preparative SDS−PAGE and subsequent electroelution. In this way about 17 nmol of homogeneous 60 kDa polypeptide was obtained from 55 nmol of R1.

Lys-C Digestion and Peptide Mapping of the 26 kDa Fragment. Prior to proteolytic digestion of the 26 kDa fragment, SDS was first removed from the protein by repeated dilutions and reconcentrations with Tris buffer containing 2 M freshly deionized urea. The protein was not carboxamidomethylated with iodoacetamide prior to proteolytic cleavage since this fragment contains only one cysteine. Treatment of the 26 kDa fragment with Lys-C resulted in complete digestion with a control showing only marginal autodigestion of Lys-C. The crude mixture was purified by RP-HPLC on a Vydac C-18 column, and 1 min fractions (1 mL) were collected in polypropylene microcentrifuge tubes (Figure 1). These were analyzed by MALDI-TOF mass spectrometry.

All of the observed peptides could be assigned to the expected Lys-C generated proteolytic fragments of the N-terminus of the R1-sequence.[3] However, one relatively abundant peptide (as judged by its UV absorption at 220 nm during HPLC purification; Figure 1) could not be assigned to any of the expected Lys-C proteolytic fragments. The molecular ion of this peptide was observed by MALDI-TOF to be 2176 ± 2 (all mass values cited refer to $[M+H]^+$ ions and are isotope-averaged, rather than monoisotopic values, unless stated otherwise). To purify this peptide to homogeneity, it was rechromatographed at pH 2 with a very shallow gradient of acetonitrile. The resulting homogeneous peptide was submitted to sequencing by automated Edman degradation resulting in the sequence ISLPTPI which cor-

[2] dATP is an allosteric effector for nucleotide reduction which binds uniquely to R1.

[3] The peptide comprising tyrosine 155 to lysine 194 was not observed, possibly due to modification of the unalkylated cysteine at position 179.

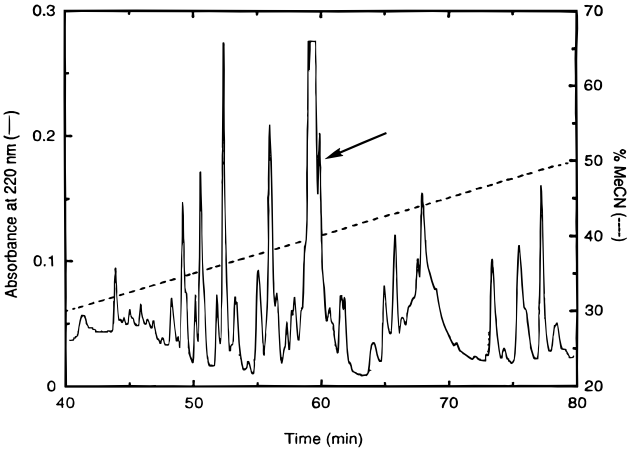


FIGURE 1: HPLC trace of the purification of the peptides generated by Lys-C digestion of the 26 kDa fragment. The peak corresponding to the C-terminal peptide is indicated with an arrow.

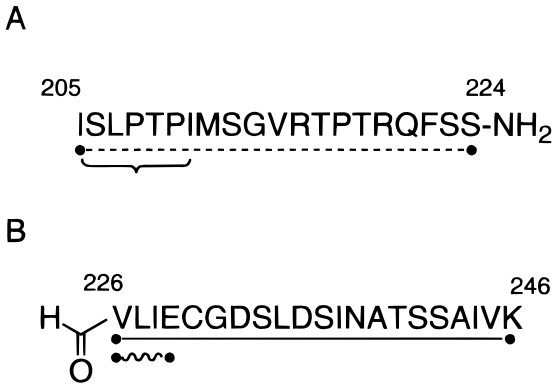


FIGURE 2: Mass spectrometric identification of selected peptides produced by (A) Lys-C digestion of the 26 kDa fragment and (B) trypsin digestion of the N-terminal CNBr fragment of the 60 kDa polypeptide. (Dotted line) Peptide identified by MALDI-TOF and FAB-MS; (Brace) peptide sequenced by Edman degradation; (solid line) peptide identified by MALDI-TOF, and (wavy line) peptide sequenced by CID-MS.

responds to residues 205−211 of R1 (Figure 2). Thus, based on the observed molecular mass of 2176 and the published protein sequence, the peptide spans residues 205−224. The mass spectrometric data suggest, therefore, that the Ser224−Ser225 bond represents the location of cleavage of the C225S mutant since this linkage does not correspond to a Lys-C recognition site. However, an alternative interpretation of the data was also considered.

Commercially available, standard grade Lys-C preparations are not completely homogeneous and can contain contaminating proteins including carboxypeptidases. It was thus considered possible that the point of fragmentation could have been several residues upstream from Ser224, and that carboxypeptidases catalyzed cleavage of a larger fragment to give the observed predominant fragment. In the case of such a scenario it would be expected that several other enzymatically truncated peptides would be observed in the digestion as well. In fact, two other peptides of low abundances (as judged by A_{220} during HPLC purification) were found with molecular ions at 2088 and 2001, which correlate well with the sequences 205−223 and 205−222, respectively. Furthermore, after rechromatography at pH 6.2 of the appropriate fractions containing these peptides, the peptide with molecular mass 2001 showed an additional molecular ion at *m*/*z* 2017 that had not been observed

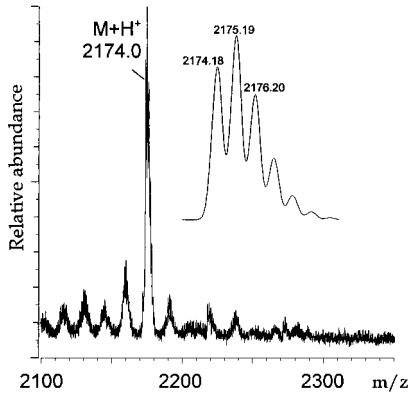


FIGURE 3: FAB mass spectrum of the major C-terminal peptide of the 26 kDa fragment. The inset shows the corresponding theoretical monoisotopic distributions for the $(M+H)^+$ ion using a resolution of 3000.

Table 1: Observed and Calculated Molecular Ions (^{12}C Component) Obtained by FAB-MS of the C-Terminal Peptides Isolated after Lys-C Digestion of the 26 kDa Fragment

sequence	observed mass of $[M+H]^+$ ion	mass calc'd for COOH terminus	mass calc'd for $CONH_2$ terminus
205−224	2174.00	2175.16	2174.18
205−223	2087.93	2088.13	2087.14
205−222	2001.4	2001.10	2000.13

previously, suggesting oxidation of methionine 212 to the corresponding sulfoxide. No peptides were found with sequences beyond residue 225. Thus, these data suggest that the predominant site of cleavage is between serines 224 and 225, and that contaminating carboxypeptidases in the Lys-C preparation generate small amounts of the peptides containing serine 223 or phenylalanine 222 at their C-termini.

To obtain an exact mass of the major and minor peptides as well as to obtain structural information about the C-terminal amino acids, the masses of the three peptides were determined by fast atom bombardment (FAB) mass spectrometry, which resolved the isotopic multiplet (Figure 3). It should be noted that the most abundant species of a peptide of this molecular mass is that containing one ^{13}C atom (*m*/*z* 2175.19), while the one at *m*/*z* 2174.18 is the true monoisotopic $(M+H)^+$ ion. The FAB-MS data and the corresponding theoretical masses for the monoisotopic, ^{12}C-only ions are compared in Table 1. These data indicate that the two minor peptides have carboxylic acid termini, while the major peptide possesses a carboxamide C-terminus. The minor peptides could therefore be products of enzymatic proteolysis, while the major peptide clearly is not. These results establish unambiguously that cleavage takes place between Ser224 and Ser225, and that the C-terminus of the 26 kDa fragment is an amide moiety rather than a carboxyl group.

CNBr Digestion and Determination of the Cleavage Site of the 60 kDa Fragment. The information available from the 26 kDa peptide defines the site of fragmentation and suggests that the 60 kDa fragment starts at Ser225. To identify this N-terminal residue, the purified 60 kDa peptide obtained by electroelution from an SDS−PAGE gel was treated with cyanogen bromide, and the resulting peptides were purified by RP-HPLC using a Vydac C-4 column. Mass spectrometric analysis (MALDI-TOF) of the peptides revealed a fraction containing a peptide of mass 8812. This is in the range of the estimated mass for the N-terminal

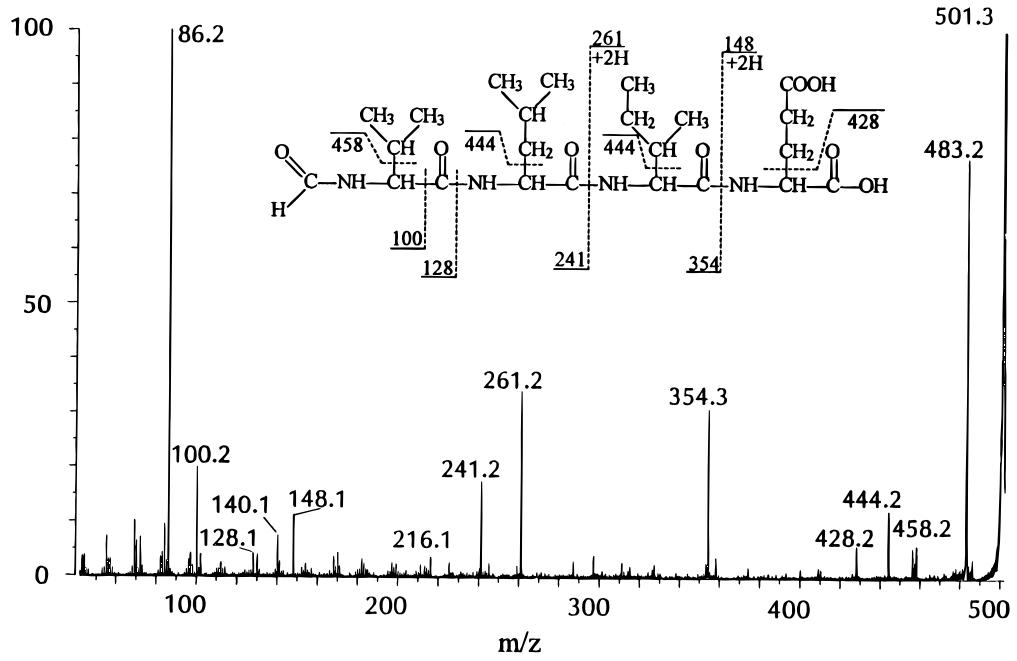


FIGURE 4: FAB CID mass spectrum of *VLIE. The $(M+H)^+$ ion was observed at 501.3. The fragment ions at 458.2 and 128.1 indicate that the formyl group is attached to the N-terminal valine residue. The ion at 86.2 represents the immonium ion of Leu or Ile, and the ion at 216.1 corresponds to the w_2 ion establishing that the second amino acid from the C-terminus is Ile (Biemann, 1988).

peptide of the 60 kDa fragment after cyanogen bromide cleavage at methionine 308 [mass of Ser225-Hse(lactone)-308 = 8859]. Consequently, this peptide was digested with trypsin, and the resulting peptides were separated by RP-HPLC. Analysis of the fractions using MALDI-TOF mass spectrometry established that the peptides indeed corresponded to fragments of the N-terminal part of the 60 kDa fragment with the exception of one with a mass of 2222. The peptide with the closest mass to 2222 would be Val226-Lys246 with a mass of 2194 (Figure 2). The difference of 28 mass units suggests formylation of one of the residues of the peptide. Although we were unable to obtain a sufficient amount of the peptide to determine a more accurate mass by FAB-MS, experiments described subsequently unambiguously established the structure of this peptide.

Determination of the Cleavage Site by Immunoadsorbent Chromatography. Once the site of cleavage was defined, an alternative approach to identify the N-terminus of the 60 kDa fragment using a method developed by Kyte and coworkers (Kyte et al., 1987) was undertaken. This strategy generates antibodies to the peptide of interest using standard methods and potentially allows separation of the desired peptide from all peptides generated in a total digest via one simple chromatography. In the present work, an immunoadsorbent specific for the peptide VLIE corresponding to residues 226−229 of the R1 polypeptide sequence was prepared. A conjugate of the synthetic peptide KVLIE and BSA carrier protein was utilized to produce polyclonal immunoglobins in rabbits. These antibodies were isolated by passing rabbit antiserum through a peptide affinity column prepared by coupling the peptide KVLIE to an agarose matrix. The desired immunoadsorbent was finally prepared by coupling the purified immunoglobins to agarose.

To verify the results obtained from the cyanogen bromide digestion, the mixture of intact C225SR1 and 26 and 60 kDa fragments was digested with endoproteinase Glu-C from *S. aureus*. The crude digest was subsequently passed over the immunoadsorbent, and the nonspecifically bound peptides were eluted with PBS. Specifically bound peptides were then eluted with 0.1 M H_3PO_4, pH 2.5. One major peptide was isolated, which was further purified by RP-HPLC. Total amino acid analysis of this peptide indicated that it consisted of Val, Leu, Ile, and Glu. This peptide, however, did not co-elute with synthetically prepared VLIE on RP-HPLC indicating that one of the amino acids was modified. Analysis of the peptide by FAB-MS showed a molecular mass of 501.3 confirming our earlier result that an adduct of 28 mass units is linked to the sequence Val226-Glu229. Using CID-MS, we were able to establish unambiguously that a formyl group is attached to the N-terminus of Val226 (Figure 4).

Cleavage of C225SR1 in ^2H_2O. In an attempt to address the question whether the proton on the formyl group at the N-terminus of the 60 kDa fragment is derived from solvent during the cleavage event, the reaction was carried out in ^2H_2O. After isolation of the N-terminal peptide using the immunoadsorbent method, mass spectrometric analysis indicated that both the molecular mass (501.3) and the fragmentation pattern were identical to that of the peptide obtained from the reaction performed in H_2O.

Cleavage Reaction under Anaerobic Conditions. The RDPR-catalyzed reduction of NDPs to dNDPs is postulated to proceed by a radical mechanism, in which cysteines 225 and 462 provide the reducing equivalents. The observation with the C225SR1 mutant that loss of every tyrosyl radical is accompanied by a polypeptide cleavage event (Mao et al., 1992a) suggests further that this fragmentation process might be radical initiated. The possibility of a radical-based polypeptide backbone fission is reminiscent of observations made during previous studies of two enzymes: pyruvate formate lyase (PFL) (Knappe et al., 1993), the anaerobic equivalent of pyruvate dehydrogenase, and the anaerobic ribonucleotide reductase from *E. coli* (Reichard, 1993a). Both of these proteins possess a glycyl radical that is essential for catalysis, and both proteins undergo O_2-dependent polypeptide cleavage (Wagner et al., 1992; King & Reichard,

1995). We therefore decided to examine the requirement of molecular oxygen for cleavage of C225SR1.

Two different approaches were used for this purpose. The first employed a chemical deoxygenating system using Fe-$(NH_4)SO_4$/DTT under an atmosphere of argon[4] (Ljungdahl & Andreesen, 1978; Tien et al., 1982). Control experiments with wt-RDPR established that under these conditions no loss of the essential tyrosyl radical occurred in the presence or absence of substrate, and that nucleotide reduction activity was not impaired. However, when C225SR1 and R2 were incubated with CDP under these conditions, loss of Y· was observed. SDS−PAGE analysis of the protein mixture after complete reduction of Y· (30 min), and optical densitometric analysis of the resulting Coomassie stained gel revealed about 15 ± 2% fission of the polypeptide backbone of C225SR1 (Figure 5). Control experiments in which Fe(II) and/or DTT were omitted produced 53 ± 2% and 51 ± 2% cleavage, respectively. Thus, the presence of Fe(II)/DTT decreased the cleavage efficiency about 3-fold.

A second approach to achieve anaerobiosis employed standard enzymatic oxygen scrubbing systems. Use of protocatechuate dioxygenase failed due to inactivation of the R2 subunit, as evidenced by the loss of A_{412} and the reduced ability to catalyze nucleotide reduction in the presence of R1. We then turned to the coupled glucose oxidase/catalase system. In comparison with an aerobic control reaction performed under identical conditions except that glucose oxidase was omitted, the cleavage efficiency of the anaerobic reaction was decreased about 2.5-fold (data not shown), in good agreement with the Fe(II)/DTT data.[5]

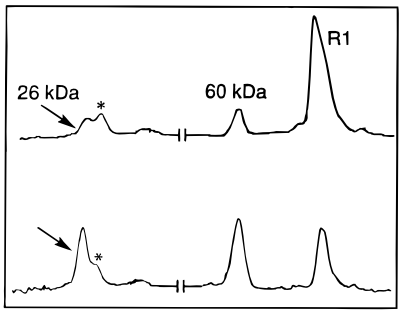


FIGURE 5: Optical densitometry performed on 10% SDS−polyacrylamide gel lanes containing the cleavage products obtained under different reaction conditions. (Top panel) Scan of the products of the cleavage reaction in the presence of 10 mM DTT and 0.1 mM $Fe(NH_4)_2(SO_4)_2$ under an atmosphere of argon. (Bottom panel) Scan of the products of a control cleavage reaction in the presence of only 10 mM DTT. The signal corresponding to the R2 subunit is removed for clarity, and the asterisk marks a signal from an impurity in the R2 preparation. A similar result was obtained from the anaerobic cleavage experiment in the presence of glucose oxidase/catalase except that additional bands from these enzymes were visible (data not shown).

DISCUSSION

The mass spectrometric analysis of the C-terminal peptide of the 26 kDa fragment and the N-terminal peptide of the 60 kDa fragment unambiguously establish the site of cleavage and the molecular structures of the fragmentation products. These results have allowed us to define the location of a residue within the active site and to address the type of chemistry involved in nucleotide reduction.

Location of the Active Site of E. coli RDPR. Biochemical studies (Lin et al., 1987) and site-directed mutagenesis (Åberg et al., 1989; Mao et al., 1989, 1992a−c) were used initially to address the question of which residues are present in the active site of RDPR from *E. coli*. The results of these studies were the basis for the working hypothesis that cysteine 439 of R1 is located on the β-face of the nucleotide substrate and is responsible for 3′-hydrogen atom abstraction, while cysteines 225 and 462 are located at the α-face and provide the actual reducing equivalents required for substrate reduction (Stubbe, 1990a; Mao et al., 1992b). Although many substrate-based analogs have been used in attempts to label and identify an active site residue, no stable adducts have been thus far isolated. The recently published high-resolution crystal structure of the R1 subunit (Uhlin & Eklund, 1994) provided strong support for this model. Cysteines 225 and 462 were present as a disulfide occupying equivalent positions on two adjacent antiparallel β-strands of an α/β-barrel domain, and cysteine 439 was situated at the tip of a loop inserted into the center of the α/β-barrel. The three cysteines were located within 6 Å of one another. While the nucleotide substrate was not present in this structure, modeling studies (Uhlin & Eklund, 1994) indicated that the dimensions of the pocket, formed on one side of the barrel by the insertion of the loop, could accommodate a nucleoside diphosphate in the appropriate orientation given the postulated role for each cysteine (Stubbe, 1990a; Mao et al., 1992b). We have now shown that incubation of C225S-R1 and R2 with CDP results in cleavage of the polypeptide backbone of R1 at position 225. We interpret these results as a direct indication that Cys225 is located at or near the active site of RDPR.

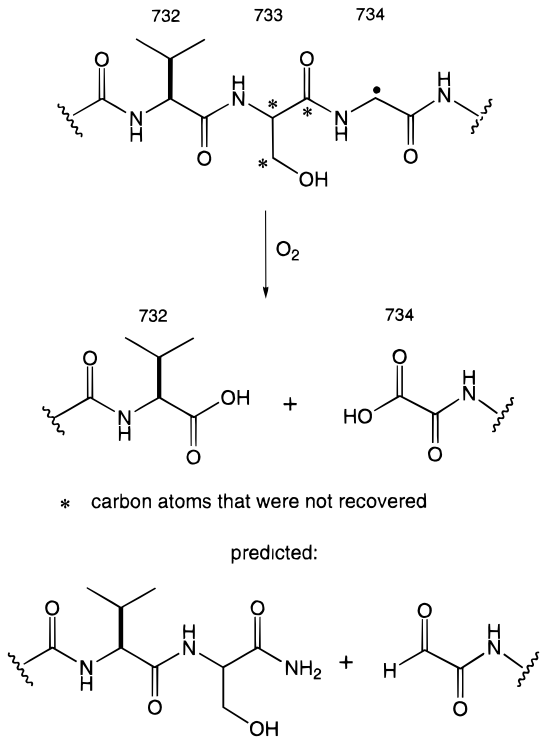
Chemistry of Polypeptide Cleavage. Given that the cleavage occurs within the active site region of R1, it was hoped that identification of the products of R1 fragmentation might provide insight into the mechanism of the nucleotide reduction process, specifically the roles of Cys225 and Cys462. The one to one correspondence between the cleavage event and tyrosyl radical loss suggested that the cleavage process is radical initiated as has been proposed for the reduction process (Stubbe, 1990b). Precedent for radical based polypeptide cleavage comes from two sources: (1) the α-amidating enzyme, a copper- and O_2-dependent enzyme that cleaves the C-terminal glycine residues of prohormones (Merckler et al., 1992), and (2) O_2-induced cleavage at the glycyl radicals of PFL and the anaerobic RNR (Wagner et al., 1992; King & Reichard, 1995).

In the α-amidating enzyme the α-hydrogen of the C-terminal glycine residue is abstracted to give an α-carbon radical which then reacts with O_2 to generate a peroxyl

[4] Although Fe(II) and DTT in the presence of O_2 can generate radicals as pointed out by one referee, it has been commonly used in anaerobic buffers in a number of enzyme systems including PFL (Knappe et al., 1984) and nitrogenase (Ljungdahl & Andreesen, 1978; Zheng & Dean, 1994). In the present work the buffers (Hepes or Tris) were first degassed prior to the addition of Fe(II)/DTT. Control experiments (see text) indicated that no polypeptide cleavage, loss of RDPR activity, or loss of the tyrosyl radical took place during this procedure, suggesting that any adventitiously generated radicals had no effect on the cleavage reaction.

[5] It should be pointed out that the overall cleavage efficiency for all reactions (aerobic and anaerobic) carried out in the presence of glucose oxidase, catalase, and 100 mM glucose was lower than for reactions carried out in their absence. We have not investigated the origin of this reduced cleavage.

Scheme 2



radical. Subsequent reduction of this species produces a C-terminal α-hydroxyglycine, which is rapidly hydrolyzed to generate an carboxamide on the penultimate peptide residue and glyoxalate. Alternatively, pulse radiolysis studies on model peptides have shown that an α-peroxyl radical can directly lead to loss of $O_2^{\bullet-}$ or, subsequent to reduction, peroxide, generating an acyl imine (von Sonntag, 1987), which is then hydrolyzed, once again forming glyoxalate and a C-terminal amidated peptide.

Two enzymes essential in anaerobic metabolism, PFL and RNR, have glycyl radicals required for catalysis. If the active form of these proteins is exposed to O_2, they are rapidly inactivated by a process that results in polypeptide backbone cleavage. In the case of PFL, cleavage at the glycyl radical on residue 734 led to the products shown in Scheme 2. The chemistry was suggested to be analogous to that described for the pulse radiolytic cleavage in peptides. However, an oxalylamide N-terminus was isolated rather than the predicted glyoxalamide. Knappe and co-workers postulated that the aldehyde was originally formed but was oxidized to the acid during work-up (Scheme 2). Interestingly, efforts failed to reveal the direct fate of the C-terminal cleavage product. Thus, the details of this cleavage in PFL, and whether O_2 is directly involved, remain to be established.

In the case of C225S-R1, the C-terminal fragmentation product was identified as an amide (Figure 2), analogous to that of the α-amidating enzyme and to pulse radiolysis experiments. However, the identification of the formylated valine at the N-terminus of the cleavage site and loss of two of the three carbons of Ser225 is unprecedented in the literature.[6] Several experiments were carried out in an effort to obtain mechanistic insight into the cleavage reaction occurring in R1. More efficient cleavage was observed in experiments in which the solutions were oxygenated and R1 was present in dilute concentrations, suggesting that, as in the case of PFL, the cleavage process might be O_2 induced. In addition, use of Fe(II)/DTT or glucose oxidase/catalase oxygen scrubbing systems substantially decreased the cleavage efficiency in comparison with control reactions (Figure 5). These experiments suggest that the cleavage event is at least partly O_2 dependent, but that there might also be oxygen-independent pathways leading to polypeptide cleavage.[7]

The proposed mechanisms for polypeptide fragmentation of the α-amidating enzyme and PFL both involve glycyl α-carbon radicals. In addition to considering the possibility that cleavage of C225SR1 is initiated by generation of an α-carbon radical, the alternative of initiation via a β-carbon radical on Ser225 was also considered. Such a radical could undergo a β-scission reaction cleaving the C1−C2 bond of this residue and producing an acyl radical on C1 of Ser225 and an enamine type adduct on Ser224. The acyl radical could then generate the observed formylated N-terminus by hydrogen atom abstraction from solvent or one of the cysteines in the active site. This hypothesis predicts that the formyl proton should be replaced by a deuterium if the cleavage reaction is carried out in ^2H_2O. Incubation of C225SR1 in ^2H_2O with R2 and CDP, however, gave only a protonated formyl group. This result and the unfavorable thermodynamics of such a process, cleavage of a C−C σ bond to generate a C−C π bond, suggest that the β-scission pathway is not responsible for the observed products.[8]

Thus, the studies with C225SR1 and those of the Knappe laboratory with PFL suggest that the identified products are formed by complicated multistep, radical-based processes which involve unstable intermediates that can undergo modification during work-up. While the detailed mechanism(s) of cleavage remains to be elucidated, the unusual products generated in this process provides further support for one-electron chemistry gone astray during the enzymatic reduction of nucleotides to deoxynucleotides.

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[6] Presumably, the formyl group on Val226 is derived from the C1 of Ser225, although this has not been experimentally established.

[7] At present, we cannot rule out the possibility that Fe(II)/DTT decreases cleavage efficiency by reduction of an intermediate radical.

[8] It is possible that the acyl radical abstracts a hydrogen atom from Cys439. Since the thiol hydrogen on this cysteine originates from the 3′-proton of substrate, it will not be deuterated in ^2H_2O if solvent exchange is relatively slow. Therefore, we cannot unambiguously rule out the β-scission pathway, but we think it is unlikely.

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