Location of the Redox-Active Thiols of Ribonucleotide Reductase: Sequence Similarity between the *Escherichia coli* and *Lactobacillus leichmannii* Enzymes†

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ABSTRACT: The redox-active thiols of *Escherichia coli* ribonucleoside diphosphate reductase and of *Lactobacillus leichmannii* ribonucleoside triphosphate reductase have been located by a procedure involving (1) prereduction of enzyme with dithiothreitol, (2) specific oxidation of the redox-active thiols by treatment with substrate in the absence of exogenous reductant, (3) alkylation of other thiols with iodoacetamide, and (4) reduction of the disulfides with dithiothreitol and alkylation with [1-^{14}C]iodoacetamide. The dithiothreitol-reduced *E. coli* B1 subunit is able to convert 3 equiv of CDP to dCDP and is labeled with 5.4 equiv of ^{14}C. Sequencing of tryptic peptides shows that 2.8 equiv of ^{14}C is on cysteines-752 and -757 at the C-terminus of B1, while 1.0–1.5 equiv of ^{14}C is on cysteines-222 and -227. It thus appears that two sets of redox-active dithiols are involved in substrate reduction. The *L. leichmannii* reductase is able to convert 1.1 equiv of CTP to dCTP and is labeled with 2.1 equiv of ^{14}C. Sequencing of tryptic peptides shows that 1.4 equiv of ^{14}C is located on the two cysteines of C-E-G-G-A-C-P-I-K. This peptide shows remarkable and unexpected similarity to the thiol-containing region of the C-terminal peptide of *E. coli* B1, C-E-S-G-A-C-K-I.

DNA synthesis and concomitant cell growth are dependent on the production of an adequate supply of deoxyribonucleotides by the ribonucleotide reductase catalyzed reduction of ribonucleotides. At least two distinct types of ribonucleotide reductases have been well characterized (Ashley & Stubbe, 1987). The ribonucleoside diphosphate reductase (RDPR)[1] of *Escherichia coli* requires no external cofactors and reduces nucleoside 5′-diphosphate (NDP) substrates while the ribonucleoside triphosphate reductase (RTPR) of *Lactobacillus leichmannii* requires 5′-deoxyadenosylcobalamin (AdoCbl) and reduces nucleoside triphosphate (NTP) substrates. Both types of ribonucleotide reductase require an exogenous reducing system for multiple turnovers, which can be supplied by the dithiol protein thioredoxin (Holmgren, 1985; Blakley, 1978).

These two ribonucleotide reductases differ significantly in their physical structures. RDPR is thought to consist of a 1:1 complex of two subunits, B1 and B2 (Thelander, 1973). The B1 subunit, a 175-kDa dimer of similar polypeptides, contains substrate binding sites, allosteric regulatory sites, and redox-active thiols. The B2 subunit, an 87-kDa dimer of identical polypeptides, contains a binuclear Fe(III) center associated with a stable tyrosyl radical (Sjöberg et al., 1978). The active site is thought to be at the interface of the subunits, and as only one tyrosyl radical per B2 has been observed, a half-of-the-sites reactivity model has been proposed (Sjöberg, 1986). In contrast, RTPR is a single 76-kDa polypeptide.

Despite these differences, previous studies in our laboratory have found remarkable similarities in the catalytic capabilities of RDPR and RTPR (Ashley & Stubbe, 1987). Both enzymes transiently cleave the 3′ C–H bond of substrate during reduction, with similar kinetic isotope effects being observed with 3′-^{3}H-labeled substrates along with small amounts of exchange of the 3′-hydrogen with solvent, and both are inactivated by 2′-halo-2′-deoxynucleotides by very similar if not identical mechanisms. Inactivation is accompanied by formation of a protein-bound chromophore in both cases. We now report the results of experiments that show remarkable structural similarity in the region around the RTPR and RDPR active-site dithiols, suggesting a closer relationship between these two reductases than has been previously recognized.

Materials and Methods

Enzymes. RTPR (1.5 μmol/min·mg) was purified from *L. leichmannii* (ATCC 7830) as described (Ashley et al., 1986). B1 (0.45 μmol/min·mg) was purified from *E. coli* c600/pMB1, and B2 (4.5 μmol/min·mg) was purified from *E. coli* N6405/pSPS2 as described (Salowe & Stubbe, 1986). Protein concentrations were determined spectrophotometrically by using ϵ_{280} = 101 000 M^{-1} cm^{-1} for RTPR, ϵ_{280} = 189 000 M^{-1} cm^{-1} for B1, and ϵ_{280} = 130 500 M^{-1} cm^{-1} for B2. TPCK-treated trypsin (12 500 BAEE units/mg), *Staphylococcus aureus* V8 protease (4.1 units/mg), and bovine intestinal alkaline phosphatase (1060 units/mg) were from Sigma.

General. [1-^{14}C]Iodoacetamide, [5-^{3}H]CTP, and [1-^{14}C]-CTP were from New England Nuclear. Guanidine hydrochloride was from Aldrich and was recrystallized from methanol before use. Coenzyme B_{12} was from Sigma. Di-

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[1] Abbreviations: RDPR, *Escherichia coli* ribonucleoside diphosphate reductase; RTPR, *Lactobacillus leichmannii* ribonucleoside triphosphate reductase; NDP, nucleoside 5′-diphosphate; dNDP, 2′-deoxynucleoside 5′-diphosphate; NTP, nucleoside 5′-triphosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; AdoCbl, 5′-deoxyadenosylcobalamin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SCAM, *S*-(carboxamidomethyl)cysteine; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); TPCK, *p*-tosylphenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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thiothreitol was from Chemical Dynamics. Sequanal grade trifluoroacetic acid and 6 N HCl were from Pierce. Liquid scintillation counting was performed on a Packard TriCarb 300C counter using EcoScint (National Diagnostics) scintillation cocktail. Centricon-30 ultrafiltration devices were from Amicon. HPLC was performed on a Vydac 218TP (5-μm) peptide column with a flow rate of 1.0 mL/min and UV detection at 220 nm. Solvent systems were H_2O/CH_3CN containing 0.1% CF_3COOH (system A), 10 mM ammonium acetate (pH 6.8)/CH_3CN (system B), and 20 mM potassium phosphate (pH 7.2)/CH_3CN (system C). Peptide sequencing was performed by automated Edman degradation at the University of Wisconsin Biotechnology Center. Cycles from the Edman degradation were analyzed for radioactivity by liquid scintillation counting. Enzyme prereduction was performed as previously described (Ashley et al., 1986).

RTPR Assay. A 20-μL aliquot of RTPR ($\sim$2 nmol) was added to 20 μL of 0.2 M sodium citrate, pH 6.1, 2.0 M sodium acetate, and 0.5 mM [5-^{3}H]CTP (4.08×10^6 cpm/μmol). A 1-μL portion of 4.8 mM AdoCbl was added, and the mixture was kept in the dark for 10 min at ambient temperature. The mixture was heated for 1 min in a boiling water bath and treated with 50 μL of 0.5 M Tris-HCl, pH 8.5, 20 mM $MgCl_2$, and 1.5 unit of alkaline phosphatase for 1 h at 37 °C. Deoxycytidine was then determined by the method of Steeper and Steuart (1970).

Location of RTPR Dithiols. Prereduced RTPR (48 nmol) in 480 μL of 0.1 M sodium citrate, pH 6.1, 1.0 M sodium acetate, and 5 mM EDTA was placed in a 5-mL serum-capped flask fitted with a magnetic stirring bar. Oxygen was removed from the system by three cycles of gentle evacuation (water aspirator), followed by refilling of the flask with argon. After this procedure, all additions and removals of materials were made by a syringe. A 20-μL aliquot was removed and assayed as described above. AdoCbl (20 μL, 100 nmol) and CTP (50 μL, 500 nmol) were added in the dark. After 5 min, another 20-μL aliquot was removed and assayed for residual RTPR activity. A 250-μL portion of deoxygenated 0.5 M iodoacetamide was added, and the reaction was kept in the dark for 30 min. After addition of 1.2 mL of deoxygenated 0.3 M Tris-HCl, pH 8.0, 5 mM EDTA, and 8 M guanidine hydrochloride, the reaction was kept for 3 h in the dark. The mixture was chromatographed on Sephadex G-50 (1.5 × 12 cm) with 0.3 M Tris-HCl, pH 8.0, 5 mM EDTA, 2 M guanidine hydrochloride, and 0.02% NaN_3. Fractions were monitored by absorbance at 280 nm, and protein-containing fractions were concentrated by centrifugal ultrafiltration (Centricon 30) to 1 mL. Guanidine hydrochloride (700 mg) was added, and the solution was deoxygenated as before. DTT (40 μL, 400 nmol) was added, and the mixture was stirred under argon for 1 h. [1-^{14}C]Iodoacetamide (50 μL, 2.15 μmol, 3.7×10^6 cpm/μmol) was added, and the mixture was stirred in the dark under argon for 8 h. After addition of 25 μL of 2-mercaptoethanol, the mixture was chromatographed on Sephadex G-50 as before. The protein (7.7×10^6 cpm/μmol) was dialyzed against H_2O for 4 h, lyophilized, then dissolved in 1.0 mL of 0.1 M NH_4HCO_3, pH 8.0, and 0.02% NaN_3, and treated with 40 μg of TPCK-treated trypsin. After 3 h at 37 °C, another 40 μg of TPCK-treated trypsin was added, and the digestion was allowed to proceed for an additional 3 h. The digest was acidified with 10 μL of CF_3COOH, centrifuged, and analyzed by HPLC. Initial separation used system A (0–45% CH_3CN over 90 min). The radioactive regions were separately evaporated and chromatographed with system B (0–45% CH_3CN over 90 min). A simultaneous control reaction omitting CTP gave protein of specific radioactivity 7.8×10^5 cpm/μmol.

RDPR Assay. An aliquot of RDPR ($\sim$0.2 nmol) was added to 97 μL of 50 mM HEPES, pH 7.6, 15 mM $MgSO_4$, 1 mM EDTA, 0.25 mM dTTP, and 0.122 mM [1-^{14}C]CDP (1.1×10^7 cpm/μmol). After 10 min at ambient temperature, a 50-μL portion of 0.5 M Tris-HCl, pH 8.5, containing 1 unit of alkaline phosphatase was added. After 1 h at 37 °C, deoxycytidine was determined by the procedure of Steeper and Steuart (1970).

Location of RDPR Dithiols. Prereduced B1 (40 nmol) and B2 (4 nmol) in 640 μL of 50 mM HEPES, pH 7.6, 15 mM $MgSO_4$, 1 mM EDTA, and 0.25 mM dTTP were deoxygenated with argon as described above. A 3-μL aliquot was removed and assayed as described above. CDP (256 nmol) was added, and the mixture was kept 10 min before removal of a second 3-μL aliquot for assay. The mixture was added to 2.4 mL of deoxygenated 0.4 M Tris-HCl, pH 8.5, 6 M guanidine hydrochloride, 15 mM $MgSO_4$, 1 mM EDTA, and 0.1 M iodoacetamide and kept for 3 h in the dark. The mixture was chromatographed on Sephadex G-50 (1.5 × 16 cm) with 0.1 M Tris-HCl, pH 8.0, and 2 M guanidine hydrochloride. Fractions were monitored by absorbance at 280 nm. The protein was concentrated to 0.8 mL by ultrafiltration (Centricon 30). After addition of 610 μL of 0.1 M Tris-HCl, pH 8.0, and 8.3 M guanidine hydrochloride, the mixture was deoxygenated with argon, treated with DTT (5 μL, 592 nmol), and kept for 30 min. [1-^{14}C]Iodoacetamide (182.5 μL, 18.5 μmol, 1.93×10^6 cpm/μmol) was added, and the mixture was kept for 3 h in the dark. The protein was isolated by gel filtration on Sephadex G-50 as described above and had a specific radioactivity of 1.01×10^7 cpm/μmol. The control omitting CDP gave protein with a specific radioactivity of 2.33×10^6 cpm/μmol.

The labeled protein (15 nmol) was dialyzed against H_2O, lyophilized, suspended in 1 mL of 0.1 M NH_4HCO_3, pH 8.0, and treated with 87.5 μg of TPCK-treated trypsin for 24 h at 37 °C. After lyophilization, the sample was chromatographed on HPLC (solvent system A, 0–50% CH_3CN over 75 min) in two portions. Fractions were collected every 1 min, and 100-μL aliquots were analyzed for ^{14}C by liquid scintillation counting. Four regions of ^{14}C (I, IIA, IIB, and III) were separately evaporated. Regions IIA and IIB were each chromatographed with solvent system C (0–30% CH_3CN over 45 min). In each case, a single peak of ^{14}C comigrated with a well-defined peak in the UV trace; peak IIA eluted at 13.5% CH_3CN, while peak IIB eluted at 14.5% CH_3CN. The peptides in region III were dissolved in 0.15 mL of 0.1 M NH_4HCO_3, pH 7.8, and 2 mM EDTA and treated with 0.9 μg of *Staphylococcus aureus* V8 protease at 37 °C for 5 h. The digest was analyzed by HPLC using solvent system A, giving four regions of ^{14}C. Peaks IIIA (19.5% CH_3CN) and IIIC (21.5% CH_3CN) each gave single radiolabeled peaks upon chromatography with solvent system B. A broad region of ^{14}C was observed upon chromatography of IIIB. Region IIID was not analyzed further. Purified IIA, IIB, IIIA, and IIIC and the front region of IIIB (containing 78% of the ^{14}C in region IIIB) were rechromatographed with solvent system A and sequenced.

Results and Discussion

Early work by Thelander demonstrated the ability of RDPR from *E. coli* to reduce 2.4–3 equiv of CDP in the absence of thioredoxin, with the concomitant oxidation of RDPR B1 subunit thiols to disulfides (Thelander, 1974). The reduced B1 subunit was also shown to be capable of transferring

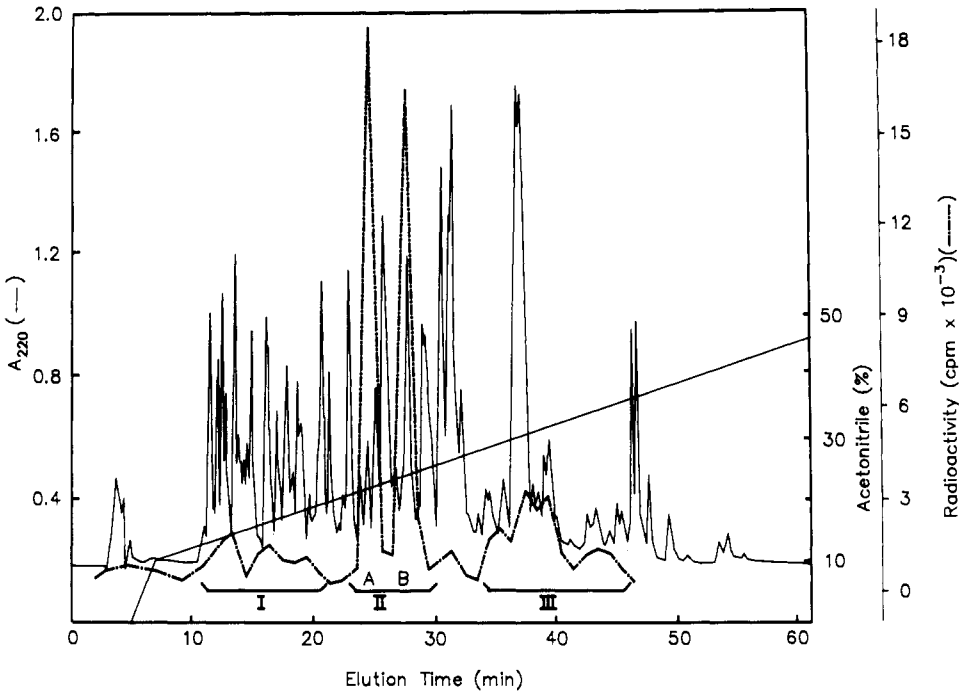

FIGURE 1: HPLC peptide map of [^{14}C]iodoacetamide-labeled RDPR. Substrate-oxidized RDPR was prepared according to the protocol given under Materials and Methods. A portion of the tryptic digest (7 nmol of RDPR) was analyzed by reversed-phase HPLC using solvent system A. Fractions were collected every 1 min, and liquid scintillation counting was performed on 100-μL aliquots of the fractions. The solid line indicates absorbance at 220 nm; the dotted line represents the ^{14}C cpm/mL in each fraction. The four regions indicated had the following ^{14}C contents: I, 0.55 equiv/B1; IIA and IIB, 2.86 equiv/B1 total; III, 2.0 equiv/B1.

electrons back into oxidized thioredoxin. On the basis of these results, Thelander proposed that redox-active dithiols were involved in the reduction of NDPs by RDPR and that thioredoxin transferred electrons into RDPR by reduction of protein disulfides. The reduction of as many as three CDPs per B1 was unexpected, given the subunit stoichiometry and the potential for half-of-the-sites reactivity. Thelander concluded, however, that the observed turnover stoichiometry must not be taken as evidence for three active sites.

The involvement of redox-active thiols on RTPR was first probed by Vitols et al. (1967). While reduction of a disulfide on RTPR upon treatment with thiols was demonstrated, they suggested that this served only to convert the enzyme into an active form and that exogenous reductant was required for substrate reduction to occur. We have subsequently shown that RTPR is capable of reducing 1 equiv of ATP in the absence of exogenous reductant (Ashley et al., 1986). Reversible electron transfer between RTPR and thioredoxin has been demonstrated (Vitols et al., 1967), so RDPR and RTPR share the ability to accept and store reducing power from exogenous reductants such as DTT and thioredoxin.

Due to the remarkable similarities in the catalytic capabilities of RDPR and RTPR, which contrast with their dissimilar cofactor requirements and structures, an experiment was designed to probe for sequence similarities in the active-site thiol regions of the two proteins. The prereduced B1 subunit of RDPR was oxidized with 6 equiv of CDP in the presence of a catalytic amount (0.1 equiv) of the B2 subunit. Before addition of CDP, an RDPR assay run in the absence of reductant indicated 3.0 ± 0.3[2] dCDP molecules produced per B1 molecule, while after oxidation only 0.08 ± 0.03 dCDP molecule was observed. Unoxidized thiols were alkylated with iodoacetamide in the presence of 6 M guanidine hydrochloride. The protein was isolated by gel filtration and treated with DTT to reduce the disulfides, and the resulting thiols were labeled with [1-^{14}C]iodoacetamide. At the conclusion of this protocol, 5.4 ± 0.6 equiv of ^{14}C were bound to the CDP-treated protein.

Tryptic peptide mapping of the alkylated B1 gave a complex set of radiolabeled peaks. Initial chromatography under acidic conditions resolved four radiolabeled regions, I (10% of the total ^{14}C), IIA and IIB (55% of the total ^{14}C), and III (38% of the total ^{14}C) (Figure 1). As the amount of labeling in region I was identical in the experiment and control, this region was considered to be an artifact of experimental procedures and was not pursued. Rechromatography of regions IIA and IIB at pH 7.2 yielded clean radiolabeled peptides, which were sequenced: IIA, D-G-A-E-D-A-Q-D-D-L-V-P-S-I-Q-D-D-G-C-E-S-G-A-C-K; IIB, D-G-A-E-D-A-Q-D-D-L-V-P-S-I-Q-D-D-G-C-E-S-G-A-C-K-I. ^{14}C was associated with both *S*-(carboxamidomethyl)cysteine (SCAM) residues in these peptides. On the basis of the revised gene sequence for B1, this peptide is the C-terminus of the 759 amino acid B1 polypeptide, residues 734–758 for IIA and residues 734–759 for IIB (J. Fuchs, personal communication).

Purification of the labeled peptides in region III was difficult, although initial sequence analysis assigned the two labeled cysteines as Cys-222 and Cys-227 in the sequence Q-F-S-S-C-V-L-I-E-C-G-D-S-L-D-S-I-N-A-T-S-S-A-I-V-K. This assignment was confirmed upon treatment of region III with *S. aureus* V8 protease. Subsequent chromatography now indicated four radiolabeled peaks, IIIA–IIID, containing 21%, 41%, 24%, and 14% of the ^{14}C in region III, respectively. Region IIID comigrated with the starting region III, suggesting that it was undigested starting material. After chromatography at pH 7.2, the following sequences were obtained: IIIA, C-G-D-S-L-D-S-I-N-A-T-S-S-A-I-V-K; IIIB, Q-F-S-S-C-V-L-I-E; IIIC, I-A-L-P-T-K-P-L-N-D-V. Peptide IIIA represents residues 227–243 and peptide IIIB residues 218–226 of B1. Together, they indicate that at least 53% of the ^{14}C in region III is located on Cys-222 and Cys-227. Although the sequencing of region IIIC did not proceed far enough to allow

[2] Values given are the averages and associated standard deviations from three repetitions of the experiment.

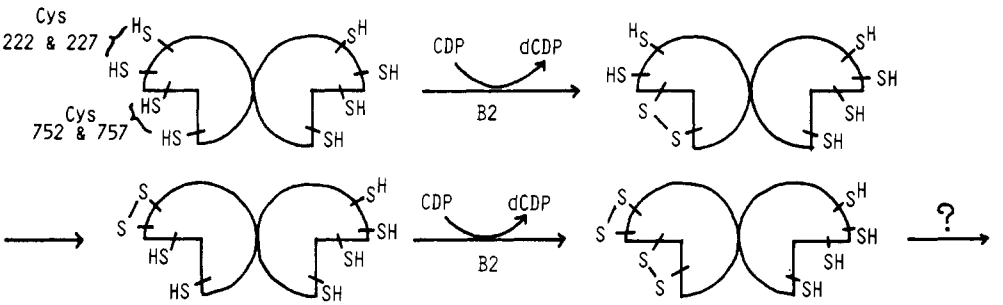

FIGURE 2: Proposed interaction of the redox-active RDPR thiols. The C-terminal dithiol Cys-752/Cys-757 reduces substrate. The Cys-222/Cys-227 dithiol subsequently transfers reducing equivalents into the active-site-dithiol by disulfide interchange.

localization of the radiolabel, analysis of the B1 gene sequence shows that the IIIc sequence represents residues 440–450, suggesting that the radiolabel in this region may be on Cys-460. Thus, 2.8 equiv of ^{14}C was on SCAM residues at positions 752 and 757 of B1, while at least 1.0 equiv of ^{14}C was on SCAM residues at positions 222 and 227. It has not been possible to determine the location of the remaining ^{14}C, although the 0.55 equiv in region I appears to be due to an experimental artifact (vide infra).

A control reaction omitting the CDP oxidation step was performed in order to probe for structural disulfides and artifacts. This control showed no change in substrate-reducing ability of RDPR during this process, reducing 3.0 ± 0.3 CDP/B1 before and after the 10-min incubation. Although no disulfide formation is predicted on the basis of this result, and previous results of Thelander (1974) suggested no structural disulfides, the control protein consistently incoporated 1.2 ± 0.1 equiv of ^{14}C. The tryptic peptides derived from the control protein showed a distribution of radioactivity similar to that observed in the experiment, with region I containing 0.44 equiv, region IIA + IIB 0.57 equiv, and region III 0.27 equiv of ^{14}C. As in the experiment, the radioactivity recovered in region II was approximately 2-fold greater than that in region III.

Although these distributions of ^{14}C were consistently observed, there are several difficulties inherent in these experiments that make interpretation difficult: (1) DTT may not completely reduce all disulfides on native oxidized RDPR; (2) some minor labeled peptides, such as those in region I, may arise from B2; (3) quantitative alkylation by iodoacetamide is unlikely; (4) tryptic digestion may be incomplete or may cleave at unexpected positions due to protease contaminants; (5) recoveries of different peptides from the HPLC may not be equivalent. Despite these limitations, the observed labeling of two distinct types of dithiol suggests hypotheses that can be verified through further experimentation using site-directed mutagenesis.

A speculative model to account for the observed production of 3 equiv of dCDP per B1 and the similar label distributions between experiments and controls is shown in Figure 2. One set of thiols functions as an electron acceptor from thioredoxin and shuttles reducing equivalents into the active-site thiols via disulfide interchange.[3] In the absence of half-of-the-sites reactivity, this model predicts that up to 4 equiv of dCDP could be produced by B1 in the absence of external reductant. DTT may be capable of providing RDPR with only 3 reducing equivalents under the prereduction conditions of our experiments. Due to the disulfide interchange, the remaining disulfide in the control experiment would be distributed between the two pairs of thiols. This disulfide interchange would preclude identification of the dithiol actually involved in substrate reduction.[4]

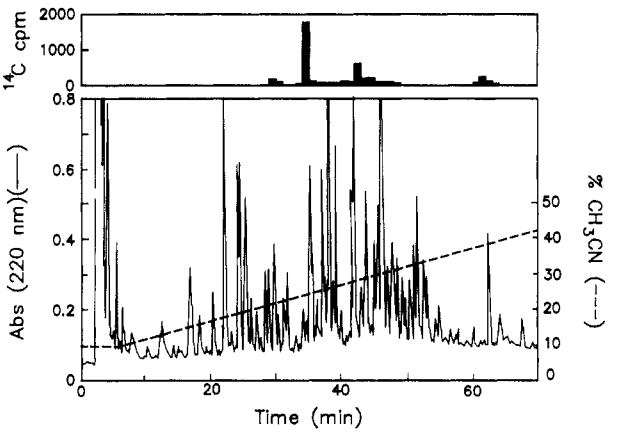

FIGURE 3: HPLC peptide map of [1-^{14}C]iodoacetamide-labeled RTPR. Substrate-oxidized RTPR was prepared and labeled according to the protocol given under Materials and Methods. A 10-nmol portion of the tryptic digest was acidified with CF_3COOH and analyzed by HPLC using solvent system A. Fractions were collected every 1 min, and liquid scintillation counting was performed on 50-μL aliquots of the fractions. The solid line indicates absorbance at 220 nm; the dashed line represents the percent CH_3CN in the elution solvent; the solid bars represent the ^{14}C cpm in a 100-μL aliquot of each fraction.

The experimental protocol used for RDPR was modified slightly for use with RTPR: prereduced RTPR was oxidized by CTP in the presence of AdoCbl, and the redox state of the protein was determined by turnover of [5-^3H]CTP. Also, all steps where AdoCbl was present were performed in darkness to avoid photolysis of the cofactor. RTPR prereduced with 30 mM DTT was allowed to react with a 5-fold excess of CTP in the presence of AdoCbl and was able to reduce 1.1 CTP per enzyme, suggesting the presence of a single pair of redox-active thiols at the active site. After treatment with CTP at pH 6.1 for 5 min in the absence of exogenous reductant, the RTPR reduced only 0.2 equiv of CTP. A control reaction, which omitted the CTP addition, lost none of its ability to reduce substrate under the experimental conditions. Upon completion of the labeling protocol, substrate-oxidized RTPR was labeled with 2.1 equiv of ^{14}C, whereas control RTPR contained only 0.2 equiv of label. These results unequivocally demonstrate for the first time the presence of a redox-active dithiol on RTPR.

The labeled RTPR was digested with TPCK-treated trypsin, and the resulting peptides were analyzed by HPLC. Two regions of radioactivity were observed in the peptide map when the digest was chromatographed under acidic conditions (Figure 3). Region I, eluting at 24% CH_3CN, comprises 67%

[3] A reviewer has suggested the possibility that disulfide interchange could be occurring between the two polypeptides of B1.

[4] Comparison of the B1 gene sequence from *E. coli*, mouse S_{49} cells, Epstein–Barr virus, and T_4 phage (R. Greenberg, personal communication) indicates that only Cys-222 is conserved (Swain & Galloway, 1986).

of the total ^{14}C, and region II, eluting at 28% CH_3CN, comprises 27% of the total ^{14}C in the digest. The remainder of the ^{14}C was distributed among several small peaks. Total recoveries of radioactivity from the HPLC were typically 50–75%. The tryptic peptide map of the control RTPR containing 0.2 equiv of ^{14}C showed the same distribution of radioactivity. The labeling in the control could represent RTPR that was not initially reduced by DTT.

Upon rechromatography at pH 6.8, region I yielded a single radiolabeled peptide eluting at 14% CH_3CN. Sequence analysis of the region I peptide by automated Edman degradation gave D-L-E-L-V-D-Q-T-D-C-E-G-G-A-C-P-I-K. Cysteine was identified as its SCAM derivative. Liquid scintillation counting of the fractions from the Edman degradation showed ^{14}C present in cycles 10 and 15. Unexpectedly, this peptide shows a high degree of similarity to the C-terminal peptide of RDPR, with only a serine to glycine change occurring in the segment between the two cysteines:

RTPR: C-E-G-G-A-C-P-I-K

RDPR: C-E-S-G-A-C-K-I

As the complete sequence of RTPR is not known, it is not possible to place this peptide within the protein. The C-terminus of RTPR has been reported to be A-L-K by carboxypeptidase analysis (Panagou et al., 1972), indicating that it is unlikely that this dithiol is at the C-terminus as is the major labeled peptide in RDPR. In related work, we have found that the cysteines of this peptide are alkylated by 2-methylene-3-(2*H*)-furanone during inactivation by the substrate analogue 2′-chloro-2′-deoxyuridine 5′-triphosphate (Ashley and Stubbe, unpublished results), thus demonstrating that these cysteines are required for RTPR activity and are thus most likely at the active site.

Rechromatography of region II at pH 6.8 yielded two peptides, with the major one eluting at 19% CH_3CN. The later eluting peak, at 28% CH_3CN, was observed to transform into the major peak upon prolonged proteolysis. Sequencing of the major peptide gave T-G-D-S-L-N-N-C-W-F, with ^{14}C associated only with the SCAM residue in cycle 8. This peptide does not show any noticeable similarity to cysteine-containing sequences in RDPR. As this peptide is produced relatively slowly during proteolysis and ends in phenylalanine, it appears to be the product of chymotrypsin contamination in the TPCK-treated trypsin. As with the minor labeled peptides from RDPR, the functional significance of this peptide is not yet known.

In summary, the redox-active thiols of the *E. coli* RDPR and of the *L. leichmannii* RTPR have been located by selective labeling with [1-^{14}C]iodoacetamide. Two pairs of thiols are involved in substrate reduction by RDPR, Cys-752/Cys-757 and Cys-222/Cys-227. The sequence of the C-terminal peptide containing the Cys-752/Cys-757 dithiol shows strong similarity to the dithiol-containing peptide isolated from RTPR, and it is thus felt that this C-terminal RDPR dithiol is directly responsible for NDP reduction. It is not certain if this sequence similarity reflects divergent evolution of RDPR and RTPR from a common precursor, which has yet to be demonstrated for two enzymes of such different physical properties and cofactor requirements, or if it is the result of convergent evolution from different precursors guided by the necessities of the chemistry used by these enzymes.

Supplementary Material Available

HPLC traces of the RDPR control and rechromatographies of each labeled peptide (9 pages). Ordering information is given on any current masthead page.

Registry No. RDPR, 9047-64-7; RTPR, 9068-66-0.

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