THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 39, Issue of September 24, pp. 40723–40728, 2004

Printed in U.S.A.

Mutations in the R2 Subunit of Ribonucleotide Reductase That Confer Resistance to Hydroxyurea*

Received for publication, March 10, 2004, and in revised form, July 9, 2004
Published, JBC Papers in Press, July 15, 2004, DOI 10.1074/jbc.M402699200

Jessica L. Sneeden and Lawrence A. Loeb‡

From the Department of Biochemistry, University of Washington, Seattle, Washington 98195

Ribonucleotide reductase is an essential enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides for use in DNA synthesis. Ribonucleotide reductase from *Escherichia coli* consists of two subunits, R1 and R2. The R2 subunit contains an unusually stable radical at tyrosine 122 that participates in catalysis. Buried deep within a hydrophobic pocket, the radical is inaccessible to solvent although subject to inactivation by radical scavengers. One such scavenger, hydroxyurea, is a highly specific inhibitor of ribonucleotide reductase and therefore of DNA synthesis; thus it is an important anticancer and antiviral agent. The mechanism of radical access remains to be established; however, small molecules may be able to access Tyr-122 directly via channels from the surface of the protein. We used random oligonucleotide mutagenesis to create a library of 200,000 R2 mutants containing random substitutions at five contiguous residues (Ile-74, Ser-75, Asn-76, Leu-77, Lys-78) that partially comprise one side of a channel where Tyr-122 is visible from the protein surface. We subjected this library to increasing concentrations of hydroxyurea and identified mutants that enhance survival more than 1000-fold over wild-type R2 at high drug concentrations. Repetitive selections yielded S75T as the predominant R2 mutant in our library. Purified S75TR2 exhibits a radical half-life that is 50% greater than wild-type R2 in the presence of hydroxyurea. These data represent the first demonstration of R2 protein mutants in *E. coli* that are highly resistant to hydroxyurea; elucidation of their mechanism of resistance may provide valuable insight into the development of more effective inhibitors.

Ribonucleotide reductase (RNR)[1] catalyzes the removal of the 2′-hydroxyl of ribonucleoside diphosphates, generating deoxyribonucleotides for use in DNA synthesis. It accomplishes reduction via a complex radical transfer mechanism that has been extensively studied (1, 2). *Escherichia coli* contains a class Ia ribonucleotide reductase similar to mammalian ribonucleotide reductases, encoded by the *nrdAB* operon (3). The enzyme functions as an $\alpha_2\beta_2$ tetramer composed of two homodimers of nonidentical subunits (4). The large subunit (R1), encoded by *nrdA*, contains the substrate binding and catalytic site as well as the allosteric effector sites. The small subunit (R2), encoded by *nrdB*, contains a diiron site that is 5 Å distant from a stable tyrosyl radical that is essential for catalysis.

Given that RNR mediates the balance of deoxynucleotide precursors for DNA synthesis, ribonucleotide reductase provides an attractive target for anticancer therapy (5). Hydroxyurea (HU) is a highly specific inhibitor of ribonucleotide reductase (6). Utilized as an anticancer agent for decades, it has more recently been investigated for its ability to enhance antiviral therapies (7, 8); presumably because its alteration of dNTP pools facilitates incorporation of chain terminating nucleoside analogs into DNA. In addition to its antiviral and anticancer properties, hydroxyurea alleviates crisis symptoms in sickle cell anemia patients through up-regulation of fetal hemoglobin (9, 10).

Hydroxyurea inhibits ribonucleotide reductase by scavenging the tyrosyl radical of the R2 subunit (11). The crystal structure of *E. coli* R2 shows that the radical is buried deep within the hydrophobic core of R2 and is inaccessible to solvent (12). This raises the question of how scavengers are able to access the radical. One hypothesis suggests that HU interacts with surface residues that conduct the radical to the surface of the protein through long range radical transfers. This hypothesis is supported by the work of Gerez and Fontecave (13) who demonstrate that hydroxylamine derivatives with large substituents are able to scavenge the tyrosine radical but would be unlikely to do so directly. Their results indicate that the charge of the scavenger is the most important property governing ribonucleotide reductase inhibition. The second hypothesis allows for scavengers, particularly small molecules like HU, to access the radical directly through small channels in the protein through which the Tyr-122 is visible in the crystal structure (14, 15). Mutants with site-specific amino acid substitutions have been shown to reduce the half-life of the radical and increase the sensitivity of RNR to scavengers (15). However, no mutants have been shown to enhance resistance and render the radical less accessible. Previous studies characterizing protein in cells resistant to hydroxyurea have all demonstrated that resistance was mediated by an increase in wild-type R2 protein levels (16–20). Specific cancers and cancer cell lines have also been shown to exhibit decreased sensitivity to hydroxyurea (17, 21). In all cases, resistance was directly correlated with an increase in expression of the wild-type R2.

In an attempt to better understand the mechanism by which hydroxyurea is able to inactivate R2, we have constructed a library of mutant R2 enzymes and selected for mutants resistant to hydroxyurea. The crystal structure shows a channel in which the phenyl ring of Tyr-122 is visible from the surface (12). This channel is partially lined by the contiguous amino acid residues 74–78 (Fig. 1). Previous studies have examined mutations at Ile-74 and Leu-77 with respect to radical content

* This work was supported by National Institutes of Health Grant RO1 CA78885. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Washington, K-056 HSB 1959 N.E. Pacific, Seattle, WA 98195. Tel.: 206-543-6015; Fax: 206-543-3967; E-mail: laloeb@u.washington.edu.

[1] The abbreviations used are: RNR, ribonucleotide reductase; HU, hydroxyurea; R2, small subunit of ribonucleotide reductase; R1, large subunit of ribonucleotide reductase.

and stability (15). Single substitutions at these hydrophobic residues effectively both reduced the radical content and rendered the RNR more susceptible to radical scavengers. We hypothesized that amino acid substitutions at these positions might alter the channel and provide a more packed structure, blocking access of scavengers to the radical. Using the method of random mutagenesis, we have obtained mutants that enhance survival of *E. coli* more than 1000-fold HU over wild-type R2 and have carried out *in vitro* studies on the accessibility of the radical to hydroxyurea.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyurea, isopropyl-1-thio-β-D-galactopyranoside, andthymidine were purchased from Sigma. *E. coli* thioredoxin reductase was the kind gift of H. Hernandez (Massachusetts Institute of Technology). Antisera to R1 and R2 were the kind gift of C. Yee (Massachusetts Institute of Technology). R2 pET expression vector was the kind gift of B. A. Brown II (Wake Forest University). Randomized oligonucleotides were obtained from Midland. Plasmid pCDNA3 and oligonucleotide primers were obtained from Invitrogen, and the pET vector was from Novagen. *E. coli* thioredoxin was purchased from Promega.

Bacterial Strains, Plasmids, and Transformations—The R2 subunit of RNR is encoded by the *nrdB* gene. R2-deficient *E. coli* strain KK446 (*F-, thr-1, leuB6, fhuA21, lacY1, glnV44(AS), lambda-, nrdB2(ts), thyA6, rpsL67(strR), thi-1, deoB37, deoC1*) obtained from the *E. coli* Genetic Stock Center (Yale University) was used in all complementation experiments (3). *E. coli* XL-1 gold (Stratagene) was used for library construction cloning. Wild-type R2 was cloned using TOPO-cloning methods (Invitrogen) from KK446. R2 was expressed in a pBR322 vector under the *lac* promoter. Bacterial transformations were carried out by electroporation (22). All R2 constructs were sequenced in entirety after cloning procedures to ensure no mutations resulted from manipulation.

Media—All cells were grown or plated in LB medium at 37 °C. KK446 is auxotrophic for thymidine; growth medium was supplemented with 20 μg/ml thymidine (3).

Library Construction and Cloning—A library of plasmids containing the R2 subunit with partially random nucleotides corresponding to amino acid positions 74–78 was assembled as described previously (23). An 86-mer oligonucleotide encompassing nucleotides 169–254 of the *nrdB* gene was synthesized (Midland): 5′-**TCC CGG G**AC CGT ATA GAT TAC CAG GCG CTG CCG GAG CAC GAA AAA CAC ATC TTT (ATC AGC AAC CTG AAA) TAT CAG ACG CT**T CTA GA**-3′. The nucleotides in parentheses were synthesized to contain 76% wild-type and 8% of each of the other three nucleotides at each position. This degree of randomness yields an average amino acid substitution of 2.38 per oligonucleotide (24). The underlined nucleotides are complementary to primers for amplification; nucleotides in boldface encode SmaI and XbaI sites, respectively. The 86-mer oligo was PCR-amplified, and PCR products were digested overnight with SmaI and XbaI at 25 °C. Digests were verified by 3% agarose gel electrophoresis and gel-purified using a Qiaquick gel extraction kit (Qiagen).

Restriction sites were engineered into the plasmid-encoded R2 by PCR amplification with site-directed mutagenic primers, DpnI digestions, and transformation (Stratagene). To minimize wild-type contamination during the ligation processes, a stuffer vector of R2 was created by replacing the wild-type region (nucleotides 223–237) with a 1100-bp fragment of DNA from pCDNA3 plasmid DNA by digestion with SmaI and XbaI. The resulting R2 stuffer vector was then digested with SmaI and XbaI, gel-purified, and ligated overnight at 12 °C to the gel-purified oligonucleotide. The ligated DNA was transformed into *E. coli*.

Complementation of E. coli KK446 and Selection of Active nrdB Mutants—Complementation was performed essentially as described previously (3). The hydroxyurea hypersensitivity of strain KK446 has been mapped to the *nrdB* locus, which encodes the R2 protein (3). Wild-type R2 on a medium-copy plasmid (pBR322) under the *lac* promoter was used as a positive control for complementation of KK446. Cells containing either stuffer, library, or wild-type plasmid-encoded R2 were diluted 1:100 from a fresh overnight culture, grown to 0.6 A_{600}, and then plated onto LB agar containing 0, 0.5, 1.0, or 2.5 mg/ml hydroxyurea and grown overnight at 37 °C.

Sequence Analysis of nrdB Mutants—Plasmid from selected mutants were isolated, retransformed into fresh KK446 cells, and retested as described above to eliminate false positives. All mutants were sequenced on Applied Biosystems model 3700 (PerkinElmer Life Sciences) using big dye terminator chemistry.

Western Blot Analysis—Whole cell extracts were prepared from *E. coli* after growth to mid-log phase, pelleted, and resuspended in 1× sample buffer (22). Samples were loaded onto SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with rabbit anti-R2 antibody and then with horseradish peroxidase-conjugated goat anti-rabbit antibody. R2 protein levels were measured by horseradish peroxidase chemiluminescent detection (Pierce). Densitometric analysis was performed using ImageQuant version 1.2 software (Amersham Biosciences).

Protein Purification—R2 proteins were expressed under the *lac* promoter and purified in apo form in pET28a+ (Novagen) with an N-terminal His tag. BL21(DE3) cells containing plasmids were diluted 1:100 from fresh overnight culture into LB medium. At 0.6 $A_{600\ nm}$, 1,10-phenanthroline was added to the culture in a final concentration of 100 μM (25). After a 15-min incubation, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside was added, and incubation was continued for 16 h at 37 °C. The expressed His_6-R2 fusion proteins were purified using a modified protocol from the supplier (Novagen). Pelleted cells from 1 liter of culture were resuspended in 15 ml of 1× binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole) with protease inhibitor mixture (Calbiochem) and 50 μg/ml egg white lysozyme. Cells were lysed by freeze-thaw cycles, RNase A was added to 10 μg/ml, and the lysed cells were centrifuged twice at 14,000 × *g* for 15 min. The resulting supernatant was applied to 2 ml of charged nickel-nitrilotriacetic acid resin and incubated for 30 min at 4 °C with slow turning. The resin was washed four times with 10 volumes of 1× binding buffer and batch-eluted with 10 ml of elution buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 200 mM imidazole). Proteins were concentrated using a 10,000 M_r concentrator (Amicon), and washed three times with 50 mM HEPES, pH 7.5, 20 mM NaCl, 10% glycerol. Purity was verified by electrophoresis through 12% SDS-PAGE. Concentration was determined by absorbance at 280 nm (extinction coefficient = 130,050 M^{-1} cm^{-1}) (26).

Reconstitution of R2—Purified apoR2 was reconstituted by the addition of a 5-fold molar excess of deoxygenated $Fe(II)(NH_4)_2(SO_4)_2$ to a deaerated protein solution that had previously been equilibrated with argon gas (27). After 15 min of incubation, R2 was exposed to air and desalted using an Amicon 10,000 M_r concentrator to remove unbound iron. Fe(II) was quantitated by the ferrozine assay (28).

Radical Half-life—Purified R2 was incubated in 50 mM HEPES, pH 7.6, 20 mM NaCl, 10 mM $MgCl_2$, and 2 mM HU, either in the presence or absence of 1.5 mM ATP, 0.5 mM CDP, and 9 μM R1 protein. The radical concentration was measured as corrected absorbance at 411 nm on a Cary 300 spectrophotometer. Absorbance values at 411 nm were applied to the following formula to obtain the radical concentration: $A_{411} - [2(A_{406}) + 3(A_{416})]/5$ = corrected A_{411} (27). The extinction coefficient of the Tyr radical is 1.784/mM × cm.

Activity Assays—The activity of wild-type and mutant R2 protein was measured essentially as described previously (29). The consumption of NADPH in a thioredoxin/thioredoxin reductase-coupled assay was measured by the decrease of absorbance at 340 nm at 37 °C. Reactions were typically carried out in 150-μl volumes containing 0.1 μM R2, 0.5 μM R1, 0.2 mM NADPH, 0.5 mM CDP, 1.6 mM ATP, 0.5 μM thioredoxin reductase, and 20 μM thioredoxin in 50 mM HEPES, pH 7.6, and 15 mM $MgSO_4$. One enzyme unit corresponds to the reduction of 1 nmol of CDP/min at 25 °C in excess of R1 protein. Each measurement represents the mean of six independent experiments.

RESULTS

R2 Library Construction—To evaluate the importance of amino acid residues 74–78 of the small subunit of ribonucleotide reductase (R2) for activity and radical stability, random sequences were substituted at nucleotides encoding these residues, resulting in a library of mutants with an average of 2 amino acid substitutions per molecule (Fig. 1) (24). Library construction is described under "Experimental Procedures." Briefly, an 86-mer oligonucleotide library containing partially random sequences was synthesized; the 15 nucleotides encoding residues 74–78 were synthesized from a mixture that contained 8% of each of the three incorrect nucleotides and 76% of the wild-type nucleotide at each position. This insert library was substituted for the corresponding wild-type sequence of R2. The resulting plasmid-encoded R2 mutant library was

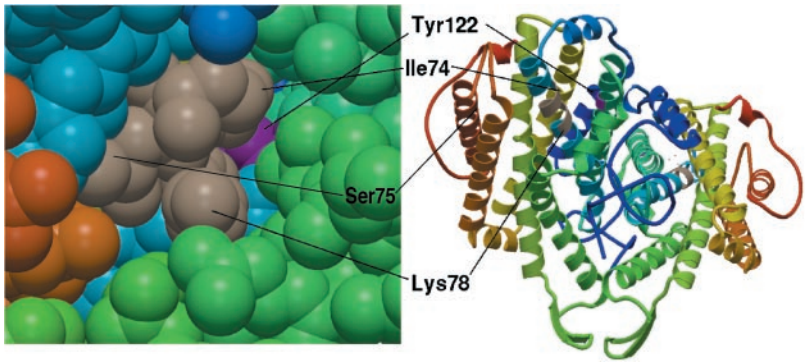

FIG. 1. **Crystal structure of *E. coli* R2 homodimer, depicting helices and space-filling model (*inset*), to illustrate channel (12, 35).** The radical-containing Tyr-122 is shown in *magenta*; residues 74–78 are *gray*.

transformed into *E. coli* KK446, a strain hypersensitive to HU (3), at an efficiency of 1.67×10^5 colonies/μg DNA.

Prior to selection, plasmid DNA was isolated from 66 clones and sequenced to determine library diversity. The number and type of substitutions are tabulated in Fig. 2*A*. The average number of amino acid substitutions per protein in the unselected library was 2.0, and the overall distribution was essentially random. Of 66 sequences, 11 contained a frameshift mutation within the randomized region and two encoded a stop codon; the library consisted of about 83% in-frame genes encoding the R2 subunit. All clones represented independent nucleotide substitutions, indicating robust library diversity (Fig 2*A*).

Selection of Resistant R2 Mutants—To select for mutant R2 proteins resistant to HU, conditions were defined under which KK446 cells expressing inactive R2 plasmids do not survive. The *E. coli* strain KK446 was characterized by its hypersensitivity to hydroxyurea, a defect that has been mapped to the *nrdB* locus, which encodes the R2 subunit (3). Although the precise mutation has not been characterized, KK446 encodes a wild-type R2 (data not shown). The phenotype can be rescued by addition of R2 in *trans* encoded on a medium-copy vector under the *lac* promoter. To select for active R2-expressing plasmids, cells containing either wild-type, random mutant library, or stuffer plasmids were plated at mid-log phase onto LB agar with concentrations of HU varying from 0 to 2.5 mg/ml. Survival was determined as the fraction of cells surviving relative to those plated without HU, as shown in Fig. 3. Complementation of the KK446 defect with plasmid-encoded wild-type R2 completely abolished HU hypersensitivity, similar to what has been described previously (3). Whereas KK446 growth is completely abolished at 0.5 mg/ml HU, cells with plasmid-encoded R2 survive at ~30% relative to cells grown in the absence of drug. Expression of plasmid-encoded stuffer vector (or pBR322 vector only, data not shown) allows minimal survival at up to 1.0 mg/ml HU. The reason for this modest survival increase relative to KK446 alone is unknown but may relate to the up-regulation of replication machinery in cells containing plasmids. The survival of cells expressing the R2 library exhibited robust complementation relative to wild-type, indicating a high proportion of active, resistant clones in the R2 library.

Plasmids from the library were isolated from individual colonies grown on 0.5 mg/ml HU and sequenced (Fig 2*B*). Of 68 sequences obtained, none contained either stop codons or frameshift mutations, indicating that at 0.5 mg/ml, all surviving colonies expressed active R2. A number of different substitutions were tolerated at residues 74, 75, and 78, but no mutations were observed at Asn-76; all substitutions at this position were silent. Leu-77 is conserved from bacterial to mammalian proteins and, as expected, tolerates only conservative substitutions, and then only at low HU concentrations (Fig 2, *B* and *C*).

S75TR2 Is Highly Resistant to HU—For identification of mutant R2 proteins highly resistant to HU, the library was selected against HU concentrations up to 2.5 mg/ml, at which concentration the wild-type plasmid-encoded R2 survives at 5.7×10^{-5} relative to that in the absence of HU (Fig. 3). Cells carrying the stuffer vector were unable to form colonies at this concentration. Plasmid DNA from the library was isolated from individual colonies and sequenced (Fig 2*C*). The sequence data reveal a striking enrichment of plasmids that contain substitutions at Ser-75. Of 78 sequences, 67 contained substitutions at this residue; 57 of these were single amino acid substitutions, 9 were double, and 1 contained a triple substitution. Of these single mutations, 41 encoded S75T substitutions. The overrepresentation of single S75T mutations was not due to a jackpot event, as all four threonine codons were observed among the 17 independent clones containing various silent mutations (data not shown). Multiple selections of the library in sequential selection at increasing concentrations of HU also yielded S75T as the predominant observed mutation. To eliminate false positives, plasmids were isolated from individual clones that grew in 2.5 mg/ml HU, and the plasmid DNA was retransformed into fresh cells and retested for resistance; all mutants exhibited no differences in survival rates (data not shown). When compared directly with the wild-type R2 plasmid in KK446, transfection with S75TR2 resulted in more than a 1000-fold increase in survival at 1.5 mg/ml HU (Fig. 3). At this concentration, less than 0.1% of cells expressing wild-type R2 survive, in contrast to cells expressing S75TR2, where 50% of cells form colonies. Concentrations higher than 2.5 mg/ml HU are required to abolish 99.9% of cells containing this mutant, which can form colonies at up to 5.0 mg/ml HU (data not shown). Among the mutants obtained by selection at 2.5 mg/ml HU, S75TR2 was the most resistant (data not shown).

To ensure that resistance was not a function of protein overexpression, R2 levels in lysates obtained from cells grown to mid-log phase were quantified by Western blot analysis (Fig. 4). Extracts from *E. coli* KK446 with plasmid only (Fig. 4, *lanes 1* and *2*) or plasmids containing wild-type R2 (*lanes 3* and *4*), stuffer vector (*lanes 5* and *6*), and S75TR2 (*lanes 7* and *8*) were examined. Densitometric analysis showed that expression levels between extracts of plasmid-encoded wild-type R2 or S75TR2 are indistinguishable; therefore resistance of S75TR2 to HU is not mediated by increased R2 expression.

Radical Content and Activity of Purified R2 Proteins—Western analysis revealed that the survival increase of S75TR2 was not mediated by overexpression of the mutant protein. To analyze the relative radical content and activity of S75TR2, His-tagged wild-type and mutant R2 apoproteins were purified to homogeneity on a nickel-nitrilotriacetic acid column and reconstituted as described under "Experimental Procedures." To assess the effect of the mutation on radical and iron content, we measured the absorbance of each between 420 and 260 nm. The tyrosyl radical of R2 has an absorbance maximum at 411 nm.

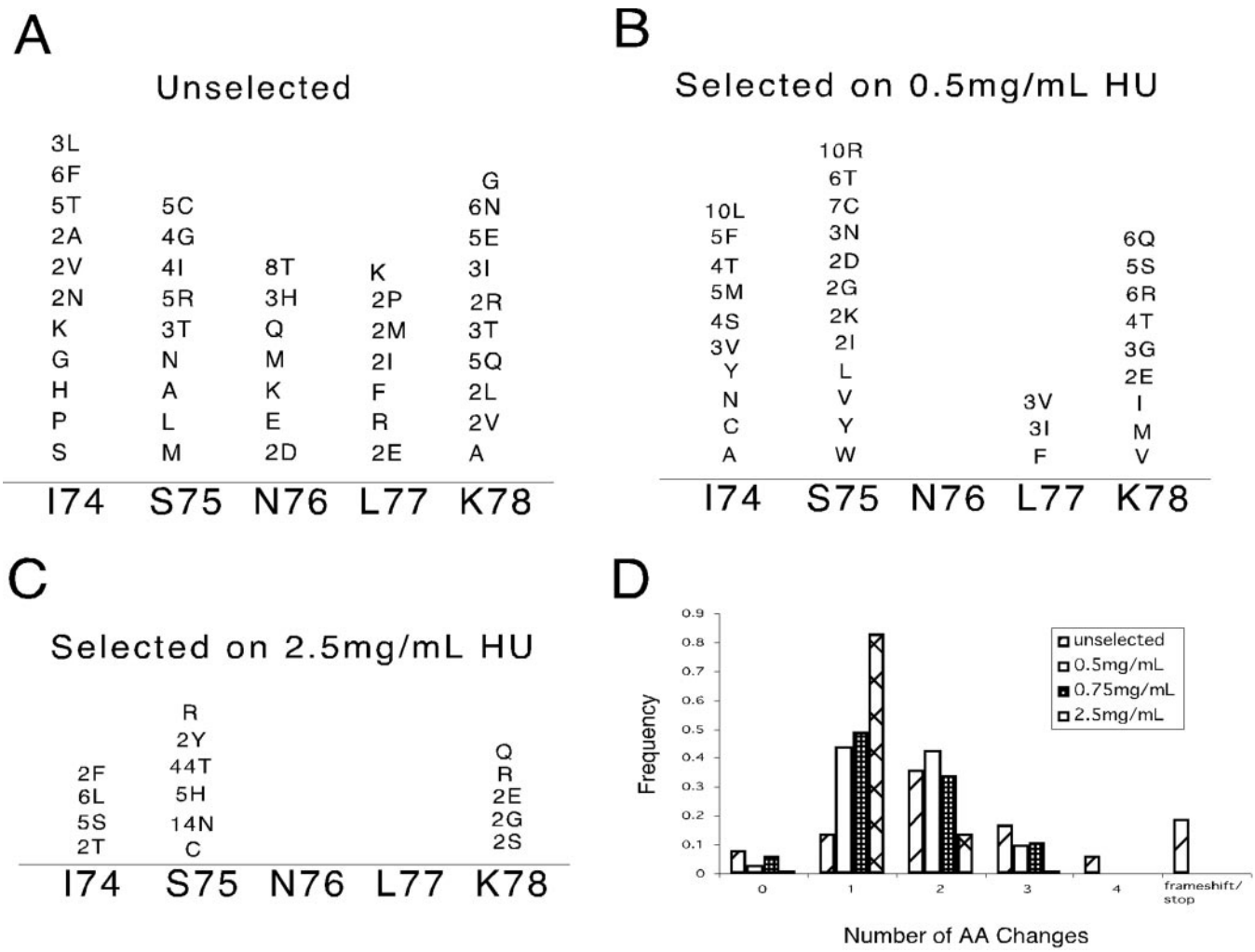

FIG. 2. **Types and frequency of amino acid substitutions.** *A*, 0 mg/ml HU; *B*, 0.5 mg/ml HU; *C*, 2.5 mg/ml HU. *D*, frequency of amino acid substitutions at varying concentrations of HU.

Reconstituted wild-type and S75T R2 exhibited no differences in radical content, with radical/dimer ratios averaging 1.2 (Table I). The absorbances at 325 and 370 nm of wild-type and mutant protein were indistinguishable, indicating a lack of change in iron center structure and content.

Ribonucleotide reductase activity was measured according to Thelander *et al.* (29) by the decrease of absorbance at 340 nm due to NADPH consumption. The specific activity of the wild-type reconstituted RNR correlates with previous observations (29, 30). The specific activity of S75TR2 is 2.6-fold less than that of the wild type (Table I).

Purified S75T R2 Demonstrates Increased HU Resistance—To assess the stability of the tyrosyl radical *in vitro*, purified R2 proteins were incubated with 2 mM HU in both the presence and absence of cofactors R1, CDP, and ATP. In the absence of HU or other scavengers, the R2 radical has an extremely long half-life, surviving for days at room temperature or for months stored at −80 °C. Scavengers like HU can efficiently abstract the radical, however, in a manner dependent on the regulatory state of the enzyme (31). As shown in Fig. 5, wild-type R2 alone had a half-life of 12.1 min at 25 °C in the presence of 2 mM HU (Fig. 5). The radical half-life of S75T R2 (12.6 min) alone was indistinguishable from that of wild-type R2. The addition of stoichiometric amounts of R1 protein to wild-type R2 did not alter the radical half-life. In contrast, the addition of R1 to the S75T mutant protein increased the radical half-life to 17.6 min in the presence of 2 mM HU. Creation of the active complex with the further addition of CDP, Mg^{2+}, and ATP reduced wild-type R2 half-life to 5.9 min, whereas S75TR2 radical half-life was only reduced to 8.4 min. For both the mutant and wild-type proteins, radical stability is reduced by 50% in the active enzyme complex. These data demonstrate that the protective effects of S75T on radical stability are mediated through the presence of R1 protein.

DISCUSSION

Ribonucleotide reductase generates deoxyribonucleotides for use in DNA synthesis. It is composed of two dissociable subunits, R1 and R2. The first free radical protein discovered, it is remarkable in that it accomplishes reduction of ribonucleoside diphosphates through a complex radical transport mechanism. The tyrosyl radical involved in catalysis resides within the hydrophobic core of the R2 subunit (12). This radical is extremely stable for days at room temperature *in vitro* (15). Because so few proteins utilize radical chemistry, ribonucleotide reductase is an attractive target for inhibition of cell proliferation; the radical is scavenged efficiently by a number of radical scavengers (2, 15), the foremost of which has been hydroxyurea.

Although the radical transport mechanism has been studied extensively (2, 32), the mechanism by which scavengers inactivate the radical remains to be established. There are two prevailing theories. Nordlund *et al.* (12) have postulated that long range electron transfers mediate radical scavenging, as

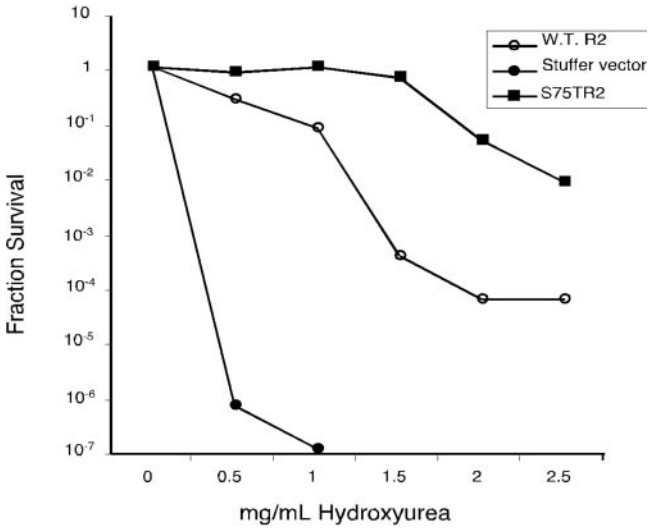

FIG. 3. **Survival curves for *E. coli* KK446 on increasing concentrations of hydroxyurea (0–2.5 mg/ml) for cells containing the following R2 constructs: wild-type (*W.T.*), stuffer vector, and S75TR2.** Survival was determined by counting the colonies of appropriate dilutions and is expressed relative to untreated cells. Each curve is the average of at least three independent experiments.

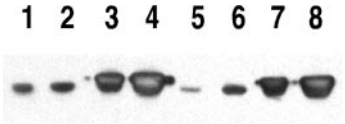

FIG. 4. **Western analysis of R2 expression in *E. coli* KK446 with or without plasmid-encoded R2 constructs.** Samples are loaded in pairs of $\sim 4 \times 10^3$ and 8×10^3 cells, respectively, on 10% SDS-PAGE. *Lanes 1* and *2*, *E. coli* KK446 alone; *lanes 3* and *4*, plasmid-encoded wild-type R2; *lanes 5* and *6*, plasmid-encoded stuffer vector; *lanes 7* and *8*, plasmid-encoded S75T R2.

TABLE I

R2	Radical content	Activity
	mol/mol R2	*Unit/mg R2*
Wild type	1.2	4380 ± 628
S75T	1.2	1663 ± 351

the Tyr-122 resides within a hydrophobic pocket 10 Å from the surface and is apparently inaccessible to solvent. The regulatory state of the enzyme affects radical stability as well, with a large decrease in stability of the active enzyme complex as opposed to R2 alone (31). These data provide support for the binding of HU to residues other than Tyr-122; indeed, multiple binding sites for HU may exist on both the R2 and R1 proteins.

The observation that mutation of small channels where Tyr-122 is visible to the protein surface results in increased sensitivity to radical scavenging (15) suggests a second theory of radical abstraction, *i.e.* that scavenging of the radical may occur through direct interaction between HU and Tyr-122 via these channels. Larsen *et al.* (14) further demonstrate that only scavengers smaller than about 4×6 Å are able to abstract the radical. Finally, the only available mouse R2 crystal structure reveals a radical accessible to solvent, with much larger channels leading to the hydrophobic core (33), and mammalian R2 proteins have demonstrated increased susceptibility to radical scavengers as compared with the corresponding *E. coli* enzyme.

Previous studies characterizing cells resistant to hydroxyurea have all demonstrated that resistance was mediated by an increase in wild-type R2 protein levels (16–20). In the present study we have used the method of random oligonucleotide mutagenesis to create R2 mutants that exhibit resistance to

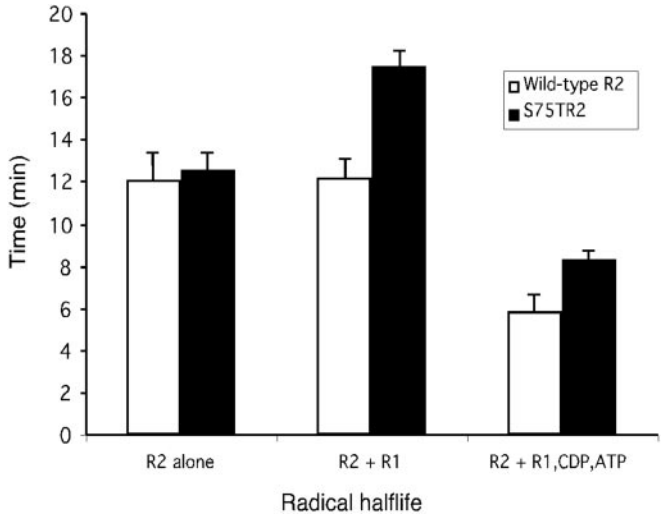

FIG. 5. **Half-life of tyrosyl radical of wild-type *versus* S75T R2 in 50 mM HEPES, pH 7.5, 20 mM NaCl with 2 mM HU.** *Left*, buffer only; *middle*, buffer, R1; *right*, buffer, 9 μM R1, 10 mM $MgCl_2$, 0.5 mM CDP, 1.5 mM ATP. The half-life was measured as the ratio of mol of tyrosyl radical to mol of R2. Radical concentration was determined from the corrected absorbance at 411 nm.

hydroxyurea. In designing our library, we reasoned that hydroxyurea might be able to abstract the radical through small channels in the protein adjacent to Tyr-122 that might be flexible enough in solution to allow direct access to the hydrophobic core. Residues that were known to participate in the reduction reaction were avoided. Amino acid residues 74–78 are located around a pocket adjacent to Tyr-122 and have not been demonstrated to participate in ribonucleotide reduction directly (Fig. 1). Of the resistant mutants that were selected, none contained substitutions at Asn-76, indicating an essential role for this residue. Asn-76 does not participate in catalysis; however, the crystal structure reveals a hydrogen bond between Asn-76 and the side chain of Ser-215 (12). In addition, the close proximity of Asn-76 (3.1 Å) to the carbonyl atom of Ser-211 suggests a possible hydrogen bond between these two residues as well. However, as activity and HU resistance are not independently selected in our system, mutations at Asn-76 may be active but exhibit a decrease in radical stability, as previously shown with mutations at Ser-211 (30). Only conservative mutations were observed at Leu-77, in concordance with its conservation across species. This residue is in van der Waals contact with Tyr-122 (12), and mutations have previously been demonstrated to affect radical stability (15). A number of mutations were isolated that conferred resistance to high concentrations of HU. Of these, the most frequent substitutions are at S75, and most of these are S75T mutations.

Spectrophotometric analysis shows that the radical/dimer ratio and iron content of purified S75TR2 are indistinguishable from wild-type R2, indicative of an intact protein. Remarkably, although the activity of the mutant enzyme is 2.6-fold less than the wild type, it confers robust resistance to HU *in vivo*. Half-life assays demonstrate that purified S75TR2 fails to confer resistance when incubated with buffer alone in the presence of HU; instead, resistance to scavenging is observed only in the presence of the R1 subunit. In contrast, the radical half-life of wild-type R2 is unaffected by the presence of R1. This suggests that upon the binding of R1 to wild-type R2 there is no change in redox potential of any binding sites that may be mediating radical transfer. It also implies that the equilibrium of radical occupation of Tyr-122 is unaffected. The addition of R1 to S75TR2 causes an increase in half-life from 12 to almost 18 min. This suggests either that a physical blocking of the chan-

nel in S75TR2 takes place upon its binding to R1 or that the mutation alters the redox potential of an alternate HU binding site, rendering this site more resistant to HU scavenging. Upon addition of CDP and ATP to the R1R2 holoenzyme, forming the active enzyme complex, both the wild type and S75TR2 half-lives show a 50% decrease. Therefore the protective effect of the mutation does not appear to be affected by enzyme catalysis.

In our selection for R2 mutants in *E. coli* KK446, we found that S75TR2 imparts a 1000-fold increase in colony formation at high HU concentrations over cells expressing wild-type R2. However, our *in vitro* data demonstrate the S75TR2 half-life to be 17.6 min, in contrast to 12 min for wild-type R2, in 2 mM HU, which is an increase of about 50%. Furthermore, the *in vitro* activity of S75TR2 is 2.6-fold less than wild-type R2. How then does S75TR2 protect cells from HU? The reason for this apparent paradox can likely be attributed to the interaction of ribonucleotide reductase with other cellular proteins by inclusion in a replication hyperstructure. Guzman *et al.* (34) show that a mutation in R1, rendering it thermolabile, demonstrates activity *in vivo* at restrictive temperatures far longer than that observed *in vitro*. They attribute the difference in activity to the participation of ribonucleotide reductase in a replication hyperstructure, or replisome. Our data indicate that the S75TR2 mutant is apparently more protective *in vivo* than *in vitro*, consistent with this mechanism. Measurement of dNTP pools *in vivo*, ongoing in our laboratory, will provide insight into this apparent difference and aid in elucidating the mechanism behind the remarkable protection conferred by S75TR2 in *E. coli*.

The crystal structure of R2 provides little information as to how the S75T mutation might function to protect the radical; no crystal structure of the R1R2 complex is available. Our data do not allow a distinction between direct and indirect access of HU to the radical; however these two hypotheses are not mutually exclusive. We postulate that when the R2 dimer is not part of an active complex, direct access is the favored reaction of radical scavengers. Upon binding to the R1 dimer, creating the holoenzyme, small conformational changes may occur in the mutant that render the radical less accessible. However, in the active enzyme complex of either mutant or wild type, the radical is more sensitive to abstraction, although the S75T mutation is still protective. This suggests that during catalysis HU is able to exploit alternative sites between Tyr-122 and the catalytic site on R1, through either direct or indirect access.

Ribonucleotide reductase is the first committed step in DNA synthesis; as such it continues to be an important target in anticancer and antiviral therapies. Our data represent the first demonstration that mutations in the coding sequence can confer protection of the tyrosyl radical of this enzyme. Elucidation of the mechanism of resistance of these mutant enzymes may provide valuable insight into the design of better inhibitors of ribonucleotide reductase for use in clinical applications.

Acknowledgments—We thank Dr. JoAnne Stubbe for R1 protein and polyclonal antiserum to R2, Dr. Elinor Adman for insightful contributions in structural analysis, and Drs. Chris Mathews and JoAnne Stubbe for critical input on this manuscript.

REFERENCES

1. Jordan, A., and Reichard, P. (1998) *Annu. Rev. Biochem.* **67,** 71–98
2. Fontecave, M. (1998) *Cell. Mol. Life Sci.* **54,** 684–695
3. Fuchs, J. A., and Karlström, H. O. (1976) *J. Bacteriol.* **128,** 810–814
4. Reichard, P., and Ehrenberg, A. (1983) *Science* **221,** 514–519
5. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. (1991) *J. Med. Chem.* **34,** 1879–1884
6. Atkin, C. L., Thelander, L., Reichard, P., and Lang, G. (1973) *J. Biol. Chem.* **248,** 7464–7472
7. Mayhew, C. N., Phillips, J. D., Greenberg, R. N., Birch, N. J., Elford, H. L., and Gallicchio, V. S. (1999) *Stem Cells* **17,** 345–356
8. Lori, F., Malykh, A., Cara, A., Sun, D., Weinstein, J. N., Lisziewicz, J., and Gallo, R. C. (1994) *Science* **266,** 801–805
9. Ho, J. A., Pickens, C. V., Gamcsik, M. P., Colvin, O. M., Ware, R. E., and Gamscik, M. P. (2003) *Exp. Hematol.* **31,** 586–591
10. Baliga, B. S., Pace, B.S., Chen, H.-H., Shah, A.K., and Yang, Y.-M. (2000) *Am. J. Hematol.* **65,** 227–233
11. Ehrenberg, A., and Reichard, P. (1972) *J. Biol. Chem.* **247,** 3485–3488
12. Nordlund, P., Sjöberg, B. M., and Eklund, H. (1990) *Nature* **345,** 593–598
13. Gerez, C., and Fontecave, M. (1991) *Biochemistry* **31,** 780–786
14. Larsen, I. K., Sjöberg, B.-M., and Thelander, L. (1982) *Eur. J. Biochem.* **125,** 75–81
15. Gerez, C., Elleingand, E., Kauppi, B., Eklund, H., and Fontecave, M. (1997) *Eur. J. Biochem.* **249,** 401–407
16. Wright, J. A., Alam, T. G., McClarty, G. A., Tagger, A. Y., and Thelander, L. (1987) *Somat. Cell Mol. Genet.* **13,** 155–165
17. Amara, F. M., Hurta, R. A., Huang, A., and Wright, J. A. (1995) *Cancer Res.* **55,** 4503–4506
18. Lye, L.-F., Hsieh H.-T., Su, K.-E., and Lee, S. T. (1997) *Mol. Biochem. Parasitol.* **90,** 353–358
19. Gräslund, A., Ehrenberg, A., and Thelander, L. (1982) *J. Biol. Chem.* **257,** 5711–5715
20. Tagger, A. Y., and Wright, J. A. (1988) *Int. J. Oncol.* **42,** 760–766
21. Burton, R. R., Kashour, T., Wright, J. A., and Amara, F. M. (2003) *Int. J. Oncol.* **22,** 21–31
22. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning*: *A Laboratory Manual*, p. 16.54, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Sneeden, J. L., and Loeb, L. A. (2003) in *Methods in Molecular Biology* (Arnold, F. H., and Georgiou, G., eds) Vol. 231, pp. 65–73, Humana Press, Totowa, NJ
24. Suzuki, M., Christians, F. C., Kim, B., Skandalis, A., Black, M. E., and Loeb, L. A. (1996) *Molecular Diversity* **2,** 111–118
25. Parkin, S. E., Chen, S., Ley, B. A., Mangravite, L., Edmondson, D. E., Huynh, B. H., and Bollinger, J. M., Jr. (1998) *Biochemistry* **37,** 1124–1130
26. Salowe, S. P., and Stubbe, J. (1986) *J. Bacteriol.* **165,** 363–366
27. Bollinger, J. M., Jr., Edmondson, D. E., Huynh, B. H., Filley, J., Norton, J. R., and Stubbe, J. (1991) *Science* **253,** 292–298
28. Stookey, L. L. (1970) *Anal. Chem.* **42,** 779–780
29. Thelander, L., Sjöberg, B. R., and Eriksson, S. (1978) *Methods Enzymol.* **51,** 227–237
30. Regnström, K., Åberg, A., Ormö, M., Sahlin, M., and Sjöberg, B.-M. (1994) *J. Biol. Chem.* **269,** 6355–6361
31. Karlsson, M., Sahlin, M., and Sjöberg, B.-M. (1992) *J. Biol. Chem.* **267,** 12622–12626
32. Stubbe, J. (2000) *Curr. Opin. Struct. Biol.* **10,** 731–736
33. Kauppi, B., Nielsen, B. B., Ramaswamy, S., Larsen, I. K., Thelander, M., Thelander, L., and Eklund, H. (1996) *J. Mol. Biol.* **262,** 706–720
34. Guzman, E. C., Caballero, J. L., and Jiménez-Sanchez, A. (2002) *Mol. Microbiol.* **43,** 487–495
35. Martz, E. (2002) *Trends Biochem. Sci.* **27,** 107–109