The active site residue Asn-437 in protein R1 of the *Escherichia coli* ribonucleotide reductase makes a hydrogen bond to the 2'-OH group of the substrate. To elucidate its role(s) during catalysis, Asn-437 was engineered by site-directed mutagenesis to several other side chains (Ala, Ser, Asp, Gln). All mutant proteins were incapable of enzymatic turnover but promoted rapid protein R2 tyrosyl radical decay in the presence of the *k*_cat inhibitor 2'-azido-2'-deoxy-CDP with similar decay rate constants as the wild-type R1. These results show that all Asn-437 mutants can perform 3'-H abstraction, the first substrate-related step in the reaction mechanism. The most interesting observation was that three of the mutant proteins (N437A/S/D) behaved as suicidal enzymes by catalyzing a rapid tyrosyl radical decay also in reaction mixtures containing the natural substrate CDP. The suicidal CDP-dependent reaction was interpreted to suggest elimination of the substrate's protonated 2'-OH group in the form of water, a step that has been proposed to drive the 3'-H abstraction step. A furanone-related chromophore was formed in the N437D reaction, which is indicative of stalling of the reaction mechanism at the reduction step. We conclude that Asn-437 is essential for catalysis but not for 3'-H abstraction. We propose that the suicidal N437A, N437S, and N437D mutants can also catalyze the water elimination step, whereas the inert N437Q mutant cannot. Our results suggest that Asn-437, apart from hydrogen bonding to the substrate, also participates in the reduction steps of catalysis by class I ribonucleotide reductase.

The enzyme ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides. RNR is crucial to all living organisms because the direct reduction is the only pathway for de novo synthesis of the DNA precursors. Currently three major RNR classes are known (1, 2). Despite their different subunit composition and metal and cofactor requirements, their substrate binding domains are homologous (3), and they are all considered to use radical chemistry involving a thyl radical initiating catalysis (4, 5). However, the mode of thyl radical formation as well as the details of the reaction mechanism differ between the classes, and only two cysteines in the active site are fully conserved in all classes. This study shows that an asparagine that is conserved in the active sites of class I and II RNRs is essential for catalysis.

The aerobic class Ia RNR of *Escherichia coli* is a well-characterized representative of class I RNRs and a prototype for eukaryotic, animal viral, and some eubacterial and archean RNRs (1, 2). The *E. coli* enzyme consists of two homodimeric subunits, denoted proteins R1 and R2, of known three-dimensional structures (6, 7). Protein R1 contains the substrate binding site with the catalytically essential redox-active cysteines (8), which have been identified by means of site-directed mutagenesis (9–12). Cysteines 225 and 462 interact directly to reduce the substrate, and cysteine 439 is proposed to transiently harbor a thyl radical that initiates catalysis. At position 122, the R2 protein contains a stable tyrosyl radical essential for catalysis (13) and an adjacent dinuclear iron site (6, 14). All class I RNRs have an array of conserved hydrogen-bonded residues between the active site of R1 and the tyrosyl radical of R2 (6, 7, 15). The hydrogen-bonded array has been proposed to function as a radical transfer pathway between the Tyr-122 in R2 and the Cys-439 in R1 (1, 16, 17), which has been corroborated by mutational analysis of the residues conserved in the pathway (9, 12, 13, 18–23).

The reaction mechanism of class I RNRs has been extensively studied during the last two decades. Many important steps of the reaction have been identified using isotope-labeled substrates (24, 25) and 2'-substituted substrate analogues (26–29). Scheme 1 outlines a mechanism based on the x-ray crystal structure of an R1-substrate complex (8) and theoretical studies (16, 30) and is an extension of the mechanism originally proposed by Stubbe and van der Donk (31). The essentials of the mechanism are as follows. In the initial step, a thyl radical (step 1 in Scheme 1) is formed on Cys-439 by radical transfer to Tyr-122 in R2 via the proposed radical transfer path. The thyl radical then abstracts a hydrogen from the 3'-position of the ribose forming an oxidized 3'-carbon-centered substrate radical (step 2 in Scheme 1). Water is eliminated from the 2'-position, forming a 3'-keto-radical intermediate (step 3 in Scheme 1), which is subsequently reduced by the redox-active cysteine pair Cys-225 and Cys-462 (steps 4–5 in Scheme 1). The hydrogen initially abstracted is then returned to the 3' position of the substrate by Cys-439 (step 6 in Scheme 1). Finally, the Tyr-122 radical is regenerated by radical transfer back to Cys-439. The oxidized active site cystine is then reduced by a C-terminal redox-active cysteine pair, Cys-754 and Cys-759 (9, 10), and the enzyme is ready for another turnover.

The active site region of R1 contains a few additional conserved residues, Ser-224, Asn-437, and Glu-441 (8). Glu-441 was recently shown to be essential for catalysis and was also shown to contribute to substrate binding (32). The study corroborates the proposed function of Glu-441 acting as a base in...
the reaction mechanism (Scheme 1) and corroborates its proposed substrate binding function seen in the R1-substrate structure (7, 8, 16, 33). Interestingly, the suicidal mutant protein E441Q allowed trapping and identification of a disulfide anion radical intermediate (34, 35), the first demonstration of one of the postulated radical intermediates of the wild-type reaction mechanism.

Asn-437 has been suggested to participate in the reduction step (8). It is also proposed to have a structural role stabilizing the loop in the active site containing Cys-439 (36) and binding the substrate 2'-OH (8). Based on structural and theoretical studies of the substrate reaction, Asn-437 and Glu-441 have been proposed to be important in the water elimination step (8, 16, 30) (Scheme 1).

In this study, several mutants of the conserved class I residues Asn-437 were constructed and characterized to elucidate the role(s) of this asparagine in the reaction mechanism of class I RNRs. Biochemical and biophysical characterization of the mutant R1 proteins shows that all mutants can perform the initial 3'-H abstraction and that Asn-437 is essential for later steps in the reaction mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides used for mutagenesis were: N437S, d(5'-GGGTAGCTCTAAGCCTGCTGGGG-3'); N437D, d(5'-GGGTAGCTCTGACGCTGGGG-3'); N437A, d(5'-GGGTAGCTCTAGCCTGGGG-3'); and N437Q, d(5'-GGGTAGCTCTAGCCTGGGG-3'). Underlining denotes mismatched nucleotide, and boldface denotes the mutant codon. These mutagenic primers were synthesized and purified by Scandinavian Gene Synthesis AB.

Restriction enzymes used were SfiI from Roche Molecular Biochemicals and MluI from Promega. The 2'-azido-2'-deoxy-CTP from U. S. Biochemical Corp. was purchased from Amersham Biosciences, Inc. Tritylamine bicarbonate buffer was prepared by titrating 0.1 triethylamine with gaseous CO₂ to pH 8.6. CDP, ATP, NADPH, benzyl-DEAE-cellulose, triethylamine, and myosin were from Sigma. dTTP (100 mU, pH 7.5) was from Amersham Biosciences, Inc., DTT was from Saveen Biotech AB. HEPES was from ICN Biomedicals Inc., and Tris-Cl was from Merck. E. coli thioredoxin and thioredoxin reductase were expressed and purified as described in Lunn et al. (37) and Russell and Model (38).

**Preparation of 2'-Azido-2'-deoxy-CDP (CaDP)—The CaDP was obtained from its triphosphate derivative (CtTP) by incubation with myosin to hydrolyze the γ-phosphate. The CaDP was separated from myosin using benzyl-DEAE anion exchange chromatography with a triethylamine bicarbonate gradient (20–650 mM) at 4–8 °C (39). Fractions were pooled and freeze-dried. The remaining triethylamine was removed by washing with methanol and evaporation for three cycles.

The CaDP was dissolved in 20 mM Tris-HCl, pH 7.6. Alternatively, the CaDP was separated from myosin by centrifugation using a Centricon filter from Amicon with 10,000-molecular weight cutoff, freeze-dried, and dissolved in 20 mM Tris-Cl, pH 7.6.

**Bacterial Strains—** E. coli CJ236 (det-1, ung-1, thi-1, relAI/pCJ105) and E. coli MV190 (Δlac-proAB), thi, supE, Δsrl-recA306::Tn10/F' traD36, proAB, lacT7ZAM15 obtained from Bio-Rad were used for mutagenesis, cloning, and plasmid preparation. E. coli SK3981 was used to produce thioredoxin and E. coli A257/pFM14 used to produce thioredoxin and cloned into wild-type pTB1 plasmid. The complete R1 gene was sequenced in pTB1 (9) containing the gene coding for protein R1 was used in combination with pGP1–2 (40) for overexpression of the mutant R1 proteins using heat induction of the T7 RNA polymerase system.

**Ligand-directed Mutagenesis—** Construction of the site-directed mutants N437S, N437A, N437D, and N437Q of pTB1 was done with the uracil-DNA method described by Kunkel et al. (41, 42) using wild-type pTB1 as a template. The Mutagene Phagemid in vitro Mutagenesis kit from Bio-Rad was used.

To verify the absence of secondary mutations, a 532-base pair SfiI/MluI fragment of the mutants N437S, N437A, and N437D was sequenced in pTB1(N437Q). In this mutant, a silent secondary mutation at position Pro-701 was found. The cultures were grown at 30 °C to induce overproduction of the cloned R1 gene. When the cultures reached stationary phase at 0.5–0.7, the temperature was raised to 42 °C to induce overproduction of the cloned R1 gene. When the cultures reached stationary phase, the cells were quickly chilled on ice and harvested by centrifugation. Pellets were frozen on dry ice and stored at −80 °C.

**Protein Purification—** Fresh cells were disintegrated in a BJOX press and resuspended in extraction buffer containing 50 mM Tris-Cl, pH 7.6, 10 mM MgCl₂, 20% glycerol, 2 mM DTT, and 10 mM phenylmethylsulfonyl fluoride. Purification was done as described by Sjoberg et al. (43) with the modifications described by Larsson et al. (44). The final purification step was ion-exchange chromatography on the fast protein liquid chromatography system from Amersham Biosciences, Inc. with a MonoQ column or the CONSEP LC100 system from Millipore with a liquid chromatography system from Amersham Biosciences, Inc. with a column of the mutants N437S, N437A, N437D, and N437Q in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10 mM MgCl₂, 20% glycerol, 200 mM imidazole, and 10 mM phenylmethylsulfonyl fluoride. Purification was monitored with SDS-PAGE with Coomassie Blue staining. The last chromatography step proved necessary to avoid unspecified precipitation of the R1 preparations during EPR experiments.

**Protein Determination—** Protein concentrations were determined using the absorbance at 280 nm minus the absorbance at 310 nm (A_280-A_310). The
stained SDS-PAGE gels were examined in a Molecular Dynamics Inc. computing laser densitometer to calculate the purity of the protein preparations. The extinction coefficients ($\varepsilon_{400-410}$) used were 180,000 M$^{-1}$ cm$^{-1}$ for protein R1 and 120,000 M$^{-1}$ cm$^{-1}$ for protein R2.

**Assay of Enzyme Activity**—The activity of ribonucleotide reductase was determined by the [$^3$H]CDP assay or the spectrophotometric assay measuring NADPH oxidation at 340 nm (26, 32), using 0.06–0.1 μM R1 and 1 μM R2 to give at least 10 × excess of R2 over R1. Reaction conditions were 0.5 mM CDP, 1.5 mM ATP, 13 mM thioredoxin, 0.5 mM thioredoxin reductase, 0.4 mM NADPH, 11 mM Mg(CH$_3$COO)$_2$ and 33 mM HEPES, pH 7.6; in the [$^3$H]CDP assay, 10 mM DTT was used as the reductant instead of thioredoxin reductase and NADPH. There was no difference in RNR-specific activity depending on the reductant used. A CDP concentration of 2.0 mM in combination with 15 mM Mg(CH$_3$COO)$_2$ was also used with other reaction conditions as above.

For measurements of protein R1 activity in crude extracts, MC1009 or MC1009/pGP1–2pTB1 were grown, harvested, and disintegrated as described above. Extracts were prepared as described above and assayed after desalting of the ammonium sulfate precipitation (43). Protein concentration was determined by the Bradford method (45), and assays were performed as described above, i.e. in the presence of an excess of protein R2.

One unit of ribonucleotide reductase activity is defined as the amount of protein R1 that catalyzes the formation of 1 nmol of product/min in the presence of excess R2 protein at 25 °C. Specific activity is expressed in units/milligrams of protein R1.

**Time-dependent UV-visible Absorption Spectroscopy**—The time dependence of tyrosyl radical decay was monitored in a PerkinElmer Life Sciences 2A scanning spectrophotometer. The enzyme mixture contained 10 μM R1 protein (mutant or wild type), 7.5 μM wild-type R2 (1.0–1.2 Tyr/R2), 2.25 mM dTTP, 15 mM Mg(CH$_3$COO)$_2$, 5 mM DTT in 50 mM Tris-Cl, pH 7.6. The reaction was started by the addition of an aliquot of CDP or CzDP to a final concentration of 2 mM or 1.7 mM, respectively, and several 300–450-nm spectra were recorded at 25 °C for 1–3 h with a speed of 480 nm/min.

The tyrosyl radical decay at 410 nm was calculated with dropline correction between 405–420 nm (i.e. the tyrosyl radical absorption at 410 nm corrected to the line defined by the absorption values at 405 and 420 nm) using the extinction coefficient ($\varepsilon_{410}$) 2,110 M$^{-1}$ cm$^{-1}$ (46). Tyrosyl radical decay curves were analyzed by curve fitting to linear, single exponential, or double exponential decay as specified in the footnotes to the tables.

The time-dependent formation of a 317-nm chromophore was monitored as above but in the absence of DTT. In these experiments, all solutions were deoxygenated by flushing with argon prior to mixing. R1 protein solutions were prepared with 10 mM DTT for 5 min at room temperature and desalted on NAP-5 columns equilibrated with argon-flushed buffer (50 mM Tris-Cl, pH 7.6, 15 mM Mg(CH$_3$COO)$_2$). At the end of incubations, all samples were analyzed by SDS-PAGE for the potential truncation of protein R1.

Stopped-flow experiments were performed in an SX.18MV BioSequentnal stopped-flow ASVD spectrofluorometer from Applied Photophysics with 55 μl of each reactant per shot. Syringe A contained 30 μM wild-type R2 (1.0–1.2 Tyr/R2) and 40 μM mutant R1 protein, and syringe B contained 4 mM CDP. Both syringes contained 0.25 mM dTTP, 15 mM Mg(CH$_3$COO)$_2$, in 50 mM Tris, pH 7.6, at 25 °C. Traces were monitored at 405, 410, 420, and 450 nm and evaluated using the SX.18MV software.

**EPR Samples and Measurements**—The reactions were performed at 25 °C by rapidly mixing equal volumes of the protein solution, 150 μM R1, 100 μM R2 in 50 mM Tris-Cl, pH 7.6, 15 mM Mg(CH$_3$COO)$_2$, 0.2–0.25 mM dTTP, 5 mM DTT, and the substrate solution of 3.34 mM CDP in the same buffer. Samples containing protein solution and buffer without substrate were used to detect the initial amount of tyrosyl radical and as a control of unspecific tyrosyl radical decay. Reactions were started by the substrate solution, and were stopped by freezing in N-pentane cooled with liquid nitrogen to −110 °C. Incubation times of 2 or longer were obtained by this method.

EPR spectra at 9 GHz measured at 77 K were recorded on a Bruker ESP 300 spectrometer using a cold finger Dewar flask for liquid nitrogen. Spin quantitation was obtained with a Cu$^{2+}$-EDTA sample (1 mM Cu$^{2+}$, 10 mM EDTA) and a secondary standard of active wild-type E. coli R2 protein (0.98 mM tyrosyl radical) by comparing the double integrals. Subtractions were performed using the ESP 300 software.

### RESULTS

**Yield and Purity of Mutant R1 Proteins**—All the mutant R1 proteins behaved as wild-type R1 throughout the protein purification procedure, and the final yields were typically 5–18 mg of R1 protein (90–95% pure)/g of wet cells, similar to earlier reported yields of active site mutants (32). The estimated amount of contaminating wild-type protein was about 1%, calculated from the amount of chromosomally encoded wild-type protein in crude extracts (Table I) (47). The overall yield throughout the purification procedure was 30–50%.

**Enzyme Activity of Mutant R1 Proteins as Compared with Wild-type R1**—An initial characterization of the mutant R1 proteins measured their specific enzyme activities (using CDP as a substrate and ATP as effector). Compared with wild-type R1 enzyme activity, all the mutants had activities of 1–2% of the wild-type activity (Table I). A low activity in a mutant protein may imply that it has some intrinsic activity per se. However, the low activities measured in the mutant proteins of this study are plausibly explained by a low amount of contaminating chromosomally encoded wild-type R1 protein (cf. Table I) (47) and an absence of intrinsic activity in the mutant proteins. The presumed absence of activity encouraged us to further characterize the reactions of the mutant proteins with the $k_{cat}$ inhibitor CzDP to monitor 3'-H abstraction from the substrate.

The mutations at position Asn-437 can promote tyrosyl radical decay in presence of CzDP—The 2'-azido-substituted substrate analogue CzDP is a mechanism-based inhibitor (Scheme 2) that mediates a half turnover reaction characterized by tyrosyl radical decay, 3'-H abstraction, and subsequent formation of a substrate-derived radical coupled to Cys-225 (step 10 in Scheme 2) (27, 48, 49). Our aim was to use the CzDP reaction to distinguish between low intrinsic activity in a mutant protein and contaminating chromosomally encoded wild-type protein in the mutant protein preparation.

Time-dependent UV-visible absorption spectroscopy was used to follow the decay of the 410-nm band in an incubation mixture containing CzDP. The time-dependent loss of the tyrosyl radical in N437Q is shown in Fig. 1 together with two controls: the wild-type reaction with a fast tyrosyl radical decay and the catalytically inactive mutant E441A (32) with no tyrosyl radical decay. It is evident that the N437Q protein produces a fast tyrosyl radical decay (Fig. 1) despite the fact that it has virtually no enzyme activity. Curve fitting of two independent exponential decay reactions to the data gave estimates of the rate constants of the tyrosyl radical decay. The N437Q mutant behaves as wild-type R1 with a fast decay of more than half of the starting concentration of tyrosyl radical in R2 (Table II). The other Asn-437 mutants were comparable with N437Q (Table II and Fig. 2A). These results show that all Asn-437
mutants can promote mechanism-based radical transfer and 
formation of a suicidal phenotype.

**All Mutations at Asn-437, Except N437Q, Have a Suicidal Phenotype**—The unexpected CzDP results prompted us to perform similar studies with the physiological substrate CDP. In the two controls (wild-type R1 and E441A), the tyrosyl radical was essentially stable over the duration of the experiment (Fig. 2B), showing that neither a fully functional radical transfer (as in wild-type R1) nor a complete lack of radical transfer (as in the E441A) gives rise to tyrosyl radical decay. The N437Q protein shows no tyrosyl radical decay (Figs. 2B and 3), in agreement with its very low enzymatic activity. In contrast, the other three mutant proteins engineered at the Asn-437 position promoted a substantial tyrosyl radical decay in the CDP reactions (Figs. 2B and 3 and Table III), suggesting that they have a suicidal phenotype. Control incubations of mutant R1 proteins with R2 and effector nucleotide but in the absence of substrate showed no decay of the tyrosyl radical during 50 min.

Based on these results, we attempted to trap radical intermediates during the suicidal reactions by performing freeze-quench EPR experiments with the N437D/S proteins. Although the tyrosyl radical decay was readily monitored in these experiments, no other radical intermediates were observed during a time span of 2 s to 30 min.

**A Furanone Adduct Is Formed in the N437D Suicidal Reaction**—Suicidal reactions with *E. coli* R1 protein often involve the formation of furanone adducts with typical chromophores in the 320-nm region if performed in the absence of DTT (50). We therefore repeated the UV-visible experiments with N437Q/A/S/D and CDP in anaerobic buffers devoid of chemical reductants to avoid trapping of the furanone by DTT. The tyrosyl radical decay rates in N437A/S/D were similar to those shown in Table III for the DTT-containing incubations. No tyrosyl radical decay occurred in the N437Q incubations. A chromophore characteristic of furanone and centered at 317 nm formed in the reactions with N437D (Fig. 4) but not in the N437Q/A/S reactions. Using molar extinction coefficients of 20,000–24,800 M⁻¹ cm⁻¹ (50) for the furanone adduct, the concentration of furanone adduct was 17–20 μM after a 40-min incubation of 10 μM N437D with excess CDP and protein R2, indicating that more than 85% of the active sites in the mutant R1 protein had a covalently bound furanone species.

**DISCUSSION**

In this study, we have assessed the importance of Asn-437, a conserved residue in the active site region in protein R1 of *E. coli* RNR. Previous studies have identified the individual roles of the conserved active site residues Cys-225, Cys-439, Cys-462 and Asn-437 in the catalytic mechanism.
Our first observation is that Asn-437 is a catalytically essential residue because four different substitutions (N437Q/A/S/D) result in catalytically inert mutant proteins. However, all mutant proteins promoted tyrosyl radical decay from protein R2 by the $k_{\text{cat}}$ inhibitor CzDP. The suicidal CzDP reaction is diagnostic for the $3^\prime$/H abstraction (Scheme 2), the first substrate-related step in the reaction sequence. The most enlightening results were that three of the mutant enzymes (N437A/S/D) also underwent rapid protein R2 tyrosyl radical decay in the presence of the natural substrate CDP. The subsequent formation of a $3^\prime$/H furanone adduct in the N437D mutant is diagnostic of substrate decomposition.

Model studies (33, 51) and theoretical calculations (16, 30) suggest that the driving force in the first part of the RNR mechanism is the elimination of the protonated $2^\prime$/H-OH group as water (Scheme 1). Subsequent one-electron reduction generates the $3^\prime$-keto intermediate (step 4 in Scheme 1), the most stable reaction intermediate in the mechanism. It has been suggested that the $3^\prime$-keto intermediate requires protonation at the keto group before the second electron reduction can occur. Reduction of the $3^\prime$-keto intermediate is therefore believed to be the rate-limiting step in single turnover conditions.

X-ray crystallography showed that Asn-437 and Glu-441 par-

### Table II

| R1 protein | $k_1$ (s$^{-1}$) | $A_1$ (relative) | $k_2$ (s$^{-1}$) | $A_2$ (relative) | $A_3$ (relative) |
|------------|----------------|-----------------|----------------|----------------|-----------------|
| Wild type$^a$ | $>0.2$ | $0.65 \pm 0.005$ | $0.003 \pm 0.0001$ | $0.30 \pm 0.004$ | $0.05 \pm 0.001$ |
| N437A$^a$ | $0.2$ | $0.65 \pm 0.007$ | $0.003 \pm 0.0001$ | $0.31 \pm 0.004$ | $0.04 \pm 0.002$ |
| N437S$^a$ | $0.2$ | $0.57 \pm 0.006$ | $0.002 \pm 0.0001$ | $0.42 \pm 0.004$ | $0.004 \pm 0.002$ |
| N437D$^a$ | $0.2$ | $0.68 \pm 0.010$ | $0.003 \pm 0.002$ | $0.18 \pm 0.06$ | $0.13 \pm 0.07$ |
| N437Q$^a$ | $0.2$ | $0.54 \pm 0.005$ | $0.006 \pm 0.0001$ | $0.44 \pm 0.004$ | $0.02 \pm 0.001$ |
| E441A$^b$ | n.d. | n.d. | n.d. | n.d. | 1.002 |

$^a$ Data were fitted to a double exponential decay, $y(t) = A_1 * e^{-k_1 t} + A_2 * e^{-k_2 t} + A_3$.

$^b$ Data were fitted to a linear equation; rel $A_3$ refers to the relative remaining signal amplitude after 50 min.
The starting radical content of 1.0–1.2 Tyr /R2 was in all experiments set to 1.00. A, amplitude; n.d., not detected.

| R1 protein    | $k_1$  | rel $A_1$ | $k_2$  | rel $A_2$ | rel $A_3$ |
|---------------|--------|-----------|--------|-----------|-----------|
| Wild type$^a$ | n.d.   |           | n.d.   |           | 0.87      |
| N437A$^b$    | 188 ± 9| 0.17 ± 0.01| 0.0004 ± 0.00001 | 0.98 ± 0.008 | 0.00 ± 0.008 |
| N437S$^b$    | 140 ± 32| 0.12 ± 0.01| 0.0006 ± 0.00001 | 0.97 ± 0.035 | 0.02 ± 0.004 |
| N437D$^b$    | n.d.   |           | 0.0003 ± 0.00001 | 0.97 ± 0.007 | 0.02 ± 0.008 |
| N437Q$^b$    | n.d.   |           | n.d.   | 1.01      |           |
| E441A$^c$    | n.d.   |           | 0.0007 ± 0.00001 | 0.28 ± 0.004 | 0.72 ± 0.005 |

$^a$ Data were fitted to a linear equation; rel $A_3$ refers to the relative remaining signal amplitude after 50 min.

$^b$ Data were fitted to a double exponential decay, $y(t) = A_1 \cdot e^{-kt_1} + A_2 \cdot e^{-kt_2} + A_3$, $k_1$, and $A_1$ were measured by stopped flow spectrophotometry and $k_2$, $A_2$, and $A_3$ by conventional scanning spectrophotometry.

$^c$ Data were fitted to a single exponential decay, $y(t) = A_2 \cdot e^{-kt_2} + A_3$.

Active Site Mutants in Ribonucleotide Reductase

Fig. 4. Furanone adduct spectrum after anaerobic incubation of N437D with CDP in the presence of protein R2. The spectrum was obtained after 40 min of incubation and subtraction of the starting spectrum before addition of the substrate.

Data were fitted to a single exponential decay,

$$y(t) = A_2 \cdot e^{-kt_2} + A_3$$

reaction sequence because no tyrosyl radical decay was observed and no diagnostic 320-nm chromophore was seen in anaerobic incubations with N437Q. Mutants N437A, N437S, and N437D conceivably allow water elimination and are likely to react according to the third reaction sequence, at least partially. A defective reduction of the 3'-keto intermediate would give rise to furanone-like reactions (32, 34) and show the diagnostic formation of the 320-nm band (52). Such a chromophore was only observed in the N437D mutant. The defective reactions in N437A/S may distribute between the second and third reaction sequence and give rise to several different end products in respectively lower concentrations. Another diagnostic test for transient non-physiological radicals formed subsequently to the water elimination step is mechanism-based truncation of the R1 protein. The suicidal mutant protein C225S plausibly forms a transient Ser-225 radical that results in truncation of the protein at that particular position (53). Neither mutant protein used in this study contained any truncated R1 (or R2) protein when assayed by SDS-PAGE after a 1–3-h incubation in the reaction mixture.

Collectively, our results demonstrate that Asn-437 is essential for catalysis in class I RNRs, plausibly by participating in the protonation and reduction of the 3'-keto intermediate (cf. Scheme 1). It seems less likely that Asn-437 is critical for the water elimination step, as suggested previously (8, 16, 30). However, a role for Asn-437 in the first one-electron reduction of the 2'-position of the substrate cannot be excluded.

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The Conserved Active Site Asparagine in Class I Ribonucleotide Reductase Is Essential for Catalysis
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