A New Mechanism-based Radical Intermediate in a Mutant R1 Protein Affecting the Catalytically Essential Glu\textsuperscript{441} in \textit{Escherichia coli} Ribonucleotide Reductase*

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Ribonucleotide reductase is an essential enzyme of all living cells and catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides. Several classes of ribonucleotide reductases with different subunit composition and cofactor requirements are known, but they all share a radical-based reaction mechanism (1).

The aerobic class Ia ribonucleotide reductase from \textit{Escherichia coli} is the best characterized enzyme. It consists of two components denoted protein R1 and protein R2, each of which is a homodimer. Protein R1 contains redox-active cysteines essential for catalysis. Cysteines 225, 439, and 462 are located at the active site, where all four physiological substrates (CDP, UDP, GDP, or ADP) can bind. R1 also contains two different allosteric sites that bind nucleoside triphosphate effector molecules. One site regulates the overall enzyme activity, and the other site determines the substrate specificity (2, 3). Protein R2 contains a stable tyrosyl free radical at position 122 and an adjacent dinuclear iron center (4–6). The tyrosyl radical is essential for catalysis.

The separate three-dimensional structures of protein R1 and of protein R2 are known (6–9). A model-built holoenzyme complex of the R1 and R2 structures indicates that the distance between the active site in R1 and Tyr\textsuperscript{122} in R2 is about 30–40 Å (8). Chains of conserved hydrogen-bonded residues leading from the active site of R1 in the direction of Tyr\textsuperscript{122} in R2, and vice versa, have been identified and are believed to be part of a radical transfer pathway between the two sites (1, 6–9). Mutation analysis of the residues postulated to be involved in radical transfer between R1 and R2 during catalysis supports this hypothesis (4, 10–14). Simpler studies have also been performed in mouse ribonucleotide reductase (15).

Recently, the three-dimensional structure of protein R1 in complex with substrate was determined (16). It shows that Cys\textsuperscript{439} is at hydrogen bonding distance to the 3'-hydrogen of the substrate nucleotide (Fig. 1A). The redox-active Cys\textsuperscript{225} and Cys\textsuperscript{462} are on the other side of the ribose moiety of the substrate nucleotide postulated to occur in the wild type reaction mechanism as well.

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The atomic coordinates (accession numbers 5R1R.pdb for E441A, 6R1R.pdb for E441D, and 7R1R.pdb for E441Q) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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hydrogen-bonded pathway to give the deoxynucleotide radical intermediate (5). The thyl radical at position 439 is then transiently regenerated by radical transfer to the 3’-position (6), and the radical is propagated to Tyr122 in protein R2. Prior to a new catalytic turnover of protein R1, the redox-active cystine at the active site must be reduced. Reduction of R1 involves yet another redox-active cysteine pair in the C-terminal part of R1 (10, 27), which interacts with the physiological reductants thioredoxin or glutaredoxin (28, 29).

The role of Glu441 is to participate in binding of the substrate, and plausibly as a general base in the reaction mechanism (8, 16) and as part of an electron transfer pathway during the reduction sequence (16). A recent study with substituted nucleosides as models for ribonucleotide reduction supports the theory that a carboxylate may act as a base in the deoxygenation at the 2’-position (30). It was also suggested that the same protonated carboxylate would act as an acid in the reduction sequence of the reaction mechanism (30).

All current evidence for substrate radical intermediates in the reaction mechanism are indirect and inferred from the observations that there is an absolute need for a stable radical and a radical transfer pathway (4, 10–14), that there is an isotope effect on the 3’-hydrogen (19, 20), that protein radicals are formed during reaction with suicidal $k_{cat}$ inhibitors (22, 24, 26), and that the polypeptide chain of an engineered R1 protein is suicidally truncated in a mechanism-based reaction sequence (18). In this study we characterize the reaction between engineered R1 proteins E441A, E441D, and E441Q and normal
substrates in presence of wild type R2. The reaction of E441D/Q and 2'-substrate analogues has been reported previously (25).

The most intriguing result of the current study is the formation of a new transient radical in a substrate-dependent reaction between E441Q and wild type protein R2. It is suggested that the new species is a substrate radical intermediate in the reaction sequence. Our current results also show that the carboxylic functionality at position 441 is absolutely essential as the E441D protein has 6–10 times lower activity than wild type R1, whereas E441A lacks enzyme activity and the amide of E441Q gives rise to a suicidal enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides used for mutagenesis were: E441A d(5'-CCTGGTGCCGGGATACC-3'), E441D d(5'-CCTGGTGCCGGAGAT-CGCC-3'), and E441Q d(5'-CCTGGTGCCGGAGATAGCCCC-3'). Underlining denotes mismatched nucleotide. These mutagenic primers were synthesized and purified by Scandinavian Gene Synthesis AB.

Restriction enzymes used were SfoI from Boehringer Mannheim and MluI from Promega.

The 2’-azido-2'-deoxy-CDP (CzDP)2 was obtained by cleavage of its CTP derivative by incubation with myosin to complete cleavage. The CzDP was separated from myosin by centrifugation using a Centricon filter from Amicon with a 10,000 M, cut-off, freeze-dried, and dissolved in 50 mM Tris-Cl, pH 7.6. The 2’-azido-2'-deoxy-CTP from U. S. Biochemical Corp. was purchased from Amersham. The myosin was purchased from Sigma.

[d-3H]CDP, [8-3H]GDP, [5-3H]CDP2 was obtained by cleavage of its CDTP by incubation with myosin to complete cleavage. The CDTP was separated from myosin by centrifugation using a Centricon filter from Amicon with a 10,000 M, cut-off, freeze-dried, and dissolved in 50 mM Tris-Cl, pH 7.6. The 2’-azido-2'-deoxy-CTP (26) from U. S. Biochemical Corp. was purchased from Amersham. The myosin was purchased from Sigma.

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concentration of free ligand, and V is the amount in moles of bound ligand/mole of protein R1.

Binding experiments with dTTP were carried out at 25 °C as described by Ormo et al. (39), using 0.5–8.0 mM tritium-labeled dTTP and R1 concentration constant at 3.2 mM or 2.9 mM. The K_v values were obtained from one experiment using seven different GDP concentrations.

GDP binding experiments were performed at 4 °C in presence of 40 μM dTTP in 50 mM Tris-Cl, pH 7.6, 15 mM Mg(CH_3COO)_2, and 2 mM DTT. Tritium-labeled GDP concentrations of 6.6–300 μM and constant R1 concentration ranging from 6.3 to 18.3 μM were used in two to eight different experiment series. Higher nucleotide concentrations were used for the mutants with higher K_v values.

EPR Samples and Measurements—The reactions were performed at 25 °C by rapidly mixing equal volumes of the protein solution, 150 mM KCl, 100 mM R2 in 50 mM Tris-Cl, pH 7.6, 15 mM Mg(CH_3COO)_2, 0.25 mM dTTP, 5 mM DTT, and the substrate solution of 3.34 mM substrate (CDP, GDP, or 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 adding the substrate solution to the protein solution and stopped by freezing in n-pentane cooled with liquid nitrogen to -110 °C. Incubation times of 2 s or longer were obtained by this method.

EPR spectra at 9 GHz measured at 77 K were recorded on a Bruker ESP 300 or Bruker 2000-SC 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 nm tyrosyl radical) by comparing the double integrals. Subtractions were performed using the ESP 300 software. The C2D-derived signal in R1 E441D and the substrate-dependent signal in E441Q were obtained by partial subtraction of the EPR spectrum of the wild type tyrosyl radical. Evaluation of the power of the half saturation, P_0, from microwave power saturation curves was performed as described by Sahlin et al. (40).

For kinetics at room temperature of E441D in reaction with CDP, the EPR spectrometer was coupled to a stopped flow assembly as described by Laussmann et al. (41). Syringe A contained 100 μM R2 and 150 μM R1, and syringe B contained 3.36 mM CDP. Both syringes contained 500 μM dTTP, 5 mM MgCl_2, 5 mM DTT in 50 mM Tris-Cl, pH 7.6. The formation of the C2D-derived signal in R1 and the tyrosyl radical decay in protein R2 were determined at a field corresponding to the maximum of the EPR first derivative line of the two studied species. The kinetic scan and the field scan were triggered by the stopped flow assembly.

Time-dependent UV-visible Absorption Spectroscopy—The time dependence of tyrosyl radical decay and formation of a 316-nm chromophore was monitored in a Perkin-Elmer A2 scanning spectrophotometer. The enzyme mixture contained 20 μM of E441Q protein, 15 μM wild type R2 (with a radical concentration of 20 μM), 0.25 mM dTTP, 15 mM Mg(CH_3COO)_2, in 50 mM Tris-Cl, pH 7.6. The reaction was started by addition of an aliquot of CDP or GDP to a final concentration of 2 mM, and 300–450-nm spectra were recorded at 25 °C for 90 min. All solutions were deoxygenated by flushing with argon prior to mixing. E441Q was prereduced with 10 mM DTT for 5 min at room temperature and R1 concentration constant at 3.2 mM. The structures of the mutant R1 proteins were solved by using the wild type structure as the initial model (8). Differences in structures compared with the wild type structure are only at the active site. The electron density at the mutated positions corresponds to the new residues. The E441Q and E441D mutations do not lead to any significant structural changes. The active site topology of the three mutations compared with wild type is seen in Fig. 1B. The glutamine side chain in E441Q has the same orientation as the glutamate side chain in the wild type structure, and differences in enzymatic properties should be due to changes in the chemical nature of that residue. Comparing the E441D structure with the substrate containing structure suggests that the interaction between Asp^{441} and the 3' hydroxyl would be significantly weaker in the mutant. However, the carboxyl of the side chain of Asp^{441} in the mutant makes the same hydrogen bond to Asn^{225} as the glutamate in the wild type, maintaining the proposed electron transfer pathway between residues Cys^{429}, Asn^{225}, and Glu/Asp^{441} (16). The mutation of Glu^{441} to Ala leads to dramatic changes at the active site. The active site has collapsed. Met^{520} has moved to the wild type position of Glu^{441} and blocks the active site. The side chain of Cys^{430} has also moved toward Met^{520}.

Interaction of the Mutant R1 Proteins with Protein R2—The interaction between R1 and R2 can be measured directly in activity assays, as described previously (48). We found that the binding of E441D to R2, with a K_m of about 0.04 μM, was about the same as that obtained for the wild type R1 protein (Table
II), indicating a similar interaction strength. For E441A, which lacks activity (see below), the interaction with R2 was measured as inhibition of the interaction between wild type R1 and R2 protein (12). The $K_c$ of 0.22 μM for E441A showed that it is able to bind protein R2 almost as well as the wild type R1 control, which in this set of experiments had an apparent $K_c$ of 0.09 μM (Table II). An attempted $K_c$ determination for E441Q indicated that also this protein was able to bind R2. However, the suicidal character of the E441Q protein, as will be discussed later, precludes an accurate determination of the interaction constant.

Interaction of the Mutant R1 Proteins with Effector and Substrate Nucleotides—The ultrafiltration assay (39) was used to measure binding of the allosteric effector dTTP to the mutant R1 proteins (Table II). All three mutant proteins bound the effector with $K_e$ values similar to the wild type protein, indicating that the effector binding sites are intact.

To test substrate binding to the mutant R1 proteins, the ultrafiltration assay by Ormör et al. (39) was used with the substrate GDP in presence of the effector dTTP at 4 °C. This substrate-effector pair gives the strongest substrate binding to wild type protein (39, 49). The $K_b$ values in Table II show that all the mutant R1 proteins can bind substrate but with weaker binding than the wild type protein. Higher dissociation constants were expected in the active site mutant proteins, as Glu441 in the wild type protein forms a hydrogen bond to the 3'-hydroxyl group of the substrate. The weakest binding is seen in E441D with a $K_b$ of about 100 μM. The mutant proteins E441Q, and E441A bind substrate about 2 times more strongly than does E441D. The wild type controls gave an average $K_b$ of 15 μM, in reasonable agreement with previously reported wild type values (39). The number of binding sites obtained were close to two for all proteins, except for the E441A mutant, which showed approximately one binding site. The low number of binding sites seen with E441A was not further investigated, but may be explained by the observation in the crystal structure that Met282 occupies part of the substrate binding site of this mutant protein (Fig. 1B). We conclude that the residue Glu441 as proposed (8, 16) contributes to substrate binding. Probably, a carboxylic acid residue of correct side chain length is needed for an optimal interaction with the substrate.

Enzyme Activity of Mutant R1 Proteins as Compared with Wild Type R1—The enzyme activity of the mutant R1 proteins in the presence of CDP as substrate and ATP as effector are compared with that of wild type R1 protein in Table III. The low activities of E441A and E441Q (~1% of wild type activity) can be explained by the small amount of chromosomally encoded wild type R1 protein present in these extracts (10, 47). The significantly higher specific activity, approximately 8% of wild type activity, found for the E441D mutant is, on the other hand, most likely intrinsic to the mutant protein. The activity of the E441D protein was also measured in presence of CDP or GDP as substrate and dTTP as effector and compared with the corresponding activity of the wild type protein. The CDP-dependent activity of the mutant protein was 10% of the wild type activity, and the GDP-dependent activity was 18% of the wild type activity.

The pH dependence of catalysis of E441D R1 was compared with that of wild type R1 activity between pH 6.1 and 8.6. The ratios between the two activities were constant over the entire pH range measured with the highest activity values around pH 8.0 (data not shown). A similar pH optimum was observed previously for the wild type protein (50). If the pH dependence of ribonucleotide reductase activity is contributed by the position 441 residue, our results indicates that a glutamic and an aspartic side chain contribute similarly.

Only the Glu → Asp Substituted Protein Has Intrinsic Enzyme Activity as Revealed in Reaction with the Substrate Analogue CsDp—To distinguish between low intrinsic activity and contaminating chromosomally encoded wild type protein in the mutant R1 proteins, we utilized the 2'-azido-substituted substrate analogue CsDp, which is a mechanism-based inhibitor. CsDp-dependent reactions are capable of a half-turnover reaction involving loss of the tyrosyl radical and appearance of a CzDP-dependent reaction intermediate (cf. Fig. 3e) located at the active site residue Cys325 (22, 25, 51).

Fig. 3 (a–d) shows the time course of the CsDp-dependent reaction of the E441D mutant protein. Traces of the diagnostic CsDp-derived radical signal are apparent as early as 15 s after the start of incubation, and the signal increases during the first 2 min, after which it slowly decays. At 10 min, a major fraction of the total remaining EPR signal is the CsDp-dependent signal. An EPR spectrum subtracted for the remainder of the tyrosyl radical signal is shown in Fig. 3d. The decay rate of the tyrosyl radical signal and the formation rate of the CsDp-dependent radical are of the same magnitude, and about 20 times slower than corresponding values for the wild type enzyme (Table III). The calculated decay constant of the CsDp-dependent radical signal was 0.08 min⁻¹, and similar to the corre-

### Table II

| R1 protein | R1-R2 complex formation | Effector binding | Substrate binding |
|------------|-------------------------|-----------------|-------------------|
|            | $K_c^{a}$ μM            | $K_e^{b}$ μM    | $K_b^{c}$ μM      |
| Wild type  | 0.036 ± 0.004           | 2.2 ± 0.14      | 15 ± 0.8          |
| E441D      | 0.035 ± 0.006           | 2.0 ± 0.12      | 90 ± 14           |
| E441Q      | NA$^d$                  | 1.7 ± 0.25      | 49 ± 6            |
| E441A      | 0.22 ± 0.013$^e$        | 2.0 ± 0.16      | 37 ± 5$^f$        |

$^a$ $V_c$ values are 1857 and 97 units/mg for wild type and E441D respectively. $^b$ The $K_c$ value for wild type R1 was 0.09 μM in these experiments. $^c$ The number of binding sites is between 1.5 and 2.1. $^d$ NA, not applicable as the assay condition for determination of $K_c$. $^e$ The $K_e$ value for wild type R1 proteins is between 1.5 and 2.1. $^f$ Standard error for $K_c$ of R2-E441A R1 complex formation was estimated using the error propagation formula.

### Table III

| R1 protein | Specific activity $K_{int}$ | Relative $k_{int}$ | CzDP-dependent reaction $k_{max}$ | Relative $k_{max}$ |
|------------|-----------------------------|--------------------|-----------------------------------|--------------------|
|            | $s^{-1}$                    | $s^{-1}$           | $s^{-1}$                          | $s^{-1}$           |
| Wild type  | 3.1$^{b}$                   | 1.00               | 0.6$^{c}$                         | 0.6$^{c}$          |
| E441D      | 0.24$^{d}$                 | 0.08               | 0.03$^{d}$                        | 0.04$^{d}$         |
| E441Q      | 0.04$^{e}$                 | 0.01               | ≤0.0001$^{f}$                     | Not observed       |
| E441A      | 0.03$^{f}$                 | 0.01               | Not observed                       | Not observed       |

$^a$ Measured in the presence of 0.5 mM CDP (i.e. substrate saturation for wild type, E441Q, and E441A) and with 1.5 mM ATP as effector. $^b$ Measured in the presence of 2 mM CDP (i.e. substrate saturation for E441D) and with 1.5 mM ATP as effector. $^c$ M. Ekberg, unpublished data. $^d$ After curve fitting to a double exponential decay, $y(t) = A_1 \times e^{-k_1 t} + A_2 \times e^{-k_2 t}$, where $A_1$ are the maximal yields and $k_1$ the decay constants, and subtraction of a CsDp-independent decay rate of 0.002 s⁻¹. $^e$ The CsDp-derived radical was fitted as an intermediate in consecutive reactions, $y(t) = A \times k_1 (k_2 - k_1) \times e^{-k_1 t} - e^{-k_2 t}$, where $A$ is the maximal yield of the intermediate, $k_1$ is rate of formation, and $k_2$ is the rate of decay of the intermediate. $^f$ Estimated from two time points and assuming single exponential decay.
Similar formation and decay rates for the CzDP-derived
reflecting that it possesses intrinsic catalytic activity. It also
E441D protein can promote mechanism-based radical transfer,
Species were also obtained by stopped flow EPR measurements
wild type R2 and the natural substrate CDP (Fig. 4). The new
EPR signal occurred when E441Q protein was incubated with
the tyrosyl radical signal and appearance of a new transient
action with E441Q and Natural Substrates—
pathway than the CzDP-dependent inactivation of the wild
type complex.

Another possibility is that the slow decay of the tyrosyl radical
this mutant protein is capable of 3

hydrogen atom abstraction.
9

9

1

3

3

2

1

UV-visible spectra were observed previously in incubations of wild type enzyme with 2'-substituted substrate analogues, and of suicidal C225S R1 protein in presence of R2 and substrate (11, 18). The substrate-derived chro-
mophore relates to R1 adducts of a highly reactive 2-methylene-3(2H)furanone intermediate (52), and is diagnostic for reactions involving suicidal decay of substrate radical intermediates formed by 39 carbon-hydrogen bond cleavage.

**DISCUSSION**

Before the structure of protein R1 was solved, the only known conserved active site residues determined essential for catalysis were the redox-active cysteines 225, 439, and 462. These were identified by systematic biochemical analysis of mutations of conserved cysteines in protein R1 (10, 11, 17, 18). By solving the structure of protein R1, the conserved active site residues including the Cys225, Cys439, and Cys462 were identified (8). Questions could now be asked about functions of other conserved residues seen in the active site and their role in the mechanism of action of ribonucleotide reductase. The substrate binding residues were initially proposed from a model building of substrate into the active site structure (8), and has recently been confirmed in the crystal structure of R1 in complex with substrate (16). One of the proposed substrate binding residues is Glu441. This residue is also proposed to participate as a base in the reaction mechanism (8, 25) (Fig. 2). Several lines of indirect evidence (reviewed in Ref. 1) support a radical-based reaction mechanism. However, the key substrate radical intermediate has not yet been identified.

In this study, site-directed mutagenesis was used to investigate the role of the conserved glutamic acid 441 in catalysis. The Glu441 was converted to alanine, aspartic acid and glutamine, respectively, to (i) abort the carboxylic group, (ii) slightly modify the access of the side chain to the substrate, and (iii) change the chemical property but not the size of the side chain. Each mutant protein contributes information to unravel the role of Glu441 in the reaction mechanism of ribonucleotide reductase.

The Glu3Ala mutation conferred an altered topography to the active site, most likely reflected in a higher binding constant. The lack of enzyme activity in the E441A protein strongly implies that the Glu441 side chain is important in catalysis, but the possibility that an altered orientation of the substrate in the active site of E441A is the immediate cause cannot be excluded. A correct orientation of substrate may be of substrate into the active site structure (8), and has recently been confirmed in the crystal structure of R1 in complex with substrate (16). One of the proposed substrate binding residues is Glu441. This residue is also proposed to participate as a base in the reaction mechanism (8, 25) (Fig. 2). Several lines of indirect evidence (reviewed in Ref. 1) support a radical-based reaction mechanism. However, the key substrate radical intermediate has not yet been identified.

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The Glu → Ala mutation conferred an altered topography to the active site, most likely reflected in a higher binding constant. The lack of enzyme activity in the E441A protein strongly implies that the Glu441 side chain is important in catalysis, but the possibility that an altered orientation of the substrate in the active site of E441A is the immediate cause cannot be excluded. A correct orientation of substrate may be
critical for initiation of the radical transfer between R1 and R2, as was first suggested by Karlsson et al. (53).

The Glu → Asp mutant protein retains a substantial enzyme activity, despite the fact that the one carbon shorter aspartate side chain causes about 6 times weaker binding of substrate compared with the wild type protein. This shows that Glu\textsuperscript{441} is an important substrate binding residue. The activity of E441D and its identical pH dependence compared with wild type R1 were expected since glutamate and aspartate are known to have similar pK\textsubscript{a} values in model peptides as well as protein (54), and are strong indications that a carboxylate function is needed at position 441 in protein R1 for ribonucleotide reduc-tase activity.

The Glu → Gln mutation has the most illuminating charac-teristics. Even though the glutamine side chain occupies the identical geometric position in the active site as the glutamate in the wild type protein, the E441Q protein is incapable of catalytic turnover. This underscores the essentiality of a car-boxylate function at position 441 for catalysis. However, our observations, that the E441Q protein in presence of natural substrates promotes tyrosyl radical decay and formation of transient radical intermediates and a furanone degradation product, show that it can bind substrate and undergo substrate initiated radical transfer between its active site and the tyrosyl radical in R2. Thus, the carboxylate function is not needed for initiation of the mechanism-based long range radical transfer reaction or for 3'-hydrogen abstraction.

The characteristics of the new transient mechanism-based EPR triplet signal in E441Q suggest that it is a free radical and that the unpaired spin has couplings to two equivalent protons. At least three possible locations may be considered for the new radical. (i) It could be located on a side chain in R2 that participates in the long range radical transfer during catalysis. However, the microwave power saturation behavior of the radical indicates that it is not in magnetic interaction with the dioiron center of R2 making this possibility unlikely. (ii) It could be on an amino acid side chain in protein R1. Radicals seen in R1 are the CzDP-derived radical (22, 24, 55) and a perthiyl radical (26), both derivatives of Cys\textsuperscript{225} in the active site. The former EPR signal is composed of a nitrogen-related triplet and a proton related 0.6 mT doublet splitting with a g\textsubscript{av} value of 2.008 (24), and the latter is composed of three discernible doublets with a splitting of 0.6 mT and a g\textsubscript{av} value of 2.03 (26). Our radical is clearly different from these two. In addition, as a preceding radical intermediate was observed, the intermedi-ate discussed here plausibly occurs further along the reaction mechanism than the radical transfer pathway. (iii) It could be on the substrate. If the new signal is a substrate radical, it could be equivalent to a natural reaction intermediate in the wild type reaction, which in the mutant protein is observable because of a slower reaction rate in E441Q. A less likely possibility is that a new reaction course could be occurring in the mutant protein, as the suicidal reaction promoted by E441Q is mechanism-based.

For a carbon-centered substrate radical, hyperfine couplings would relate to protons in β-position to the carbon with the unpaired spin (Cα), i.e. a proton on an adjacent carbon (Cβ). Such hyperfine interactions are highly dependent on geometry. The interaction is strongest when the substituent is eclipse with the half-filled p-orbital on Cα, and displays a strong angular dependence. The hyperfine splitting from a β-proton neighboring the radical can be calculated from the empirical relation \( A = B_1 + B_2 \cos^2 \theta \) (56), where \( B_1 \) is the spin density and \( \theta \) is the angle between the plane containing the p-orbital and the plane defined by the β proton and the Cα-Cβ carbons. \( B_1 \) is close to 0 mT and \( B_2 \sim 5 \) mT (57, 58). If all spin resides in the p-orbital and maximum overlap occurs (\( \theta = 0^\circ \)), we would observe a hyperfine splitting constant of \(-5 \) mT, which is much larger than the observed 1.1 mT. However, \( \theta = 60^\circ \) would give a hyperfine splitting of \(-1.25 \) mT, which is close to the observed value. With \( \rho < 1 \) and \( \theta \neq 0^\circ \), the observed EPR pattern could be achieved. A plausible substrate radical candidate would be the 3'-radical intermediate (or a 2'-radical intermediate, cf. 2–3 in Fig. 2). The crystal structure of the bound substrate in reduced R1 is compatible with 3'-endo or 2'-endo puckering (16), in which the relevant \( \theta \) angles would be \(<40^\circ \). It is therefore quite possible that the four and two hydrogens (or 3' and 1') could give rise to a 1:2:1 triplet with \(-1.1 \) mT hyperfine splitting. The observed triplet could also arise from an unpaired electron on a nitrogen, giving a 1:1:1 triplet with a distorted line shape due to immobilization in the protein. We consider this less likely since the EPR lines are too symmetric to originate from an immobilized nitrogen species and the hyperfine splitting constant does not really fit. In addition, if the coupling were due to the nitrogen introduced in the active site by the E441Q mutation, it is unlikely that a similar radical would appear in the reaction with wild type protein and the substrate 2'-fluoromethylene-CDP (cf. Fig. 3 in Ref. 25). If, on the other hand, the radical occurred at N1 in the cytosine moiety, it would most likely couple to the proton at position C5 in analogy with what has been found for irradiated crystals of cytosine and uracil (59), but there is no other equivalent carbon linked proton in the cytosine base. Instead, since an almost identical intermediate EPR signal to the one presented in Fig. 6 is obtained also with the GDP substrate, it is most likely that the radical resides in the ribose moiety of the substrate. Definite identification has to await specific isotopic labeling experiments.

Is it plausible that a substrate radical intermediate would form and be stabilized in the E441Q-dependent reaction? Formation of the furanone product is diagnostic for 3'-hydrogen abstraction in combination with a defective reduction of the 2'-position (18, 23, 60). The furanone derivative is a degradation product of the 2'-deoxygated nucleotide, suggesting that the E441Q-dependent reaction proceeds at least to 3 in Fig. 2. LENZ and Giese (30) showed that 2'-deoxygation of a model compound was subject to general base catalysis and suggested that the rate-limiting step is deprotonation of the 3'-hydroxyl group, which would be needed for efficient leaving of the 2'-hydroxyl group. In the mutant enzyme, the Glu\textsuperscript{441} side chain would still be expected to form a hydrogen bond with the 3'-hydroxyl group (cf. Fig. 1, A and B), but not to act as a proton acceptor, which means that such a rate-limiting step would be slowed down in the mutant enzyme to allow retention of a 3'-radical intermediate (2 in Fig. 2). A plausible scenario is therefore that the E441Q-dependent reaction proceeds through 1 and 2, and with a slower rate than in the wild type reaction to 3, after which degradation to a highly reactive 2-methylene-3(2H) furanone occurs that generates the furanone:protein adduct. If this scenario is correct, the E441Q reaction would be defective in forming 4, suggesting that the carboxylate at position 441 is important for efficient deprotonation of the 3'-hydroxyl group but absolutely essential for the reduction of the 2'-position. This is in good agreement with its postulated role in the transfer of redox equivalents from the redox-active Cys\textsuperscript{225/462} to the substrate (16).

The different rate constants observed in the reaction of E441Q protein and substrate in presence of protein R2 are indicative of a consecutive reaction sequence involving at least three discernible steps. First, the very rapid decay of a major fraction of the tyrosyl radical of protein R2 (\( \simeq 1.2 \) s\(^{-1} \)) matches the very rapid appearance of an (in this report uncharacter-
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