Cysteinyl and Substrate Radical Formation in Active Site Mutant E441Q of Escherichia coli Class I Ribonucleotide Reductase*

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All classes of ribonucleotide reductase are proposed to have a common reaction mechanism involving a transient cysteine thiol radical that initiates catalysis by abstracting the 3'-hydrogen atom of the substrate nucleotide. In the class Ia ribonucleotide reductase system of Escherichia coli, we recently trapped two kinetically coupled transient radicals in a reaction involving the engineered E441Q R1 protein, wild-type R2 protein, and substrate (Persson, A. L., Eriksson, M., Katterle, B., Pötsch, S., Sahlin, M., and Sjöberg, B.-M. (1997) J. Biol. Chem. 272, 31533–31541). Using isotopically labeled R1 protein or substrate, we now demonstrate that the early radical intermediate is a cysteinyl radical, possibly in weak magnetic interaction with the diiron site of protein R2, and that the second radical intermediate is a carbon-centered substrate radical with hyperfine coupling to two almost identical protons. This is the first report of a cysteinyl free radical in ribonucleotide reductase that is a kinetically coupled precursor of an identified substrate radical. We suggest that the cysteinyl radical is localized to the active site residue, Cys439, which is conserved in all classes of ribonucleotide reductase, and which, in the three-dimensional structure of protein R1, is positioned to abstract the 3'-hydrogen atom of the substrate. We also suggest that the substrate radical is localized to the 3'-position of the ribose moiety, the first substrate radical intermediate in the postulated reaction mechanism.

De novo synthesis of deoxyribonucleotides from ribonucleotides is catalyzed by the enzyme ribonucleotide reductase (RNR). All currently known classes of RNR are considered to have a common reaction mechanism based on radical chemistry (1). The RNR classes have different subunit compositions and different metal/radical cofactor requirements, but recent structural data suggest that their substrate binding domains are homologous. The proposed reaction mechanism includes formation of a transient thyl radical at the active site, which, by abstracting the 3'-hydrogen atom of the substrate, generates a transient 3'-substrate radical (1, 2). The formation of this 3'-nucleotide radical may be the critical step (3) for the subsequent steps of the reaction mechanism.

The aerobic class Ia RNR of Escherichia coli, is composed of two homodimeric components, protein R1 and protein R2, of known three-dimensional structures (4, 5). Protein R1 is the substrate binding component and Cys439 at the active site is postulated to harbor the transient thyl radical. The cysteinyl radical is thought to be generated by long range radical transfer to the R2 component, which harbors a stable oxidized tyrosyl radical in close proximity to a diiron-oxo site. The radical transfer presumably occurs via a specific route involving at least 6 residues in R2 (Tyr122, Asp84, His118, Asp237, Trp98, Tyr95) and 3 residues in R1 (Tyr97, Tyr705, Cys439). These residues have been conserved in class I RNRs (1), which are found in all studied eukaryotes (except Euglena), and in some archaea and bacteria (6).

The in vitro kcat for the aerobic class Ia E. coli RNR is about 10 s⁻¹ (1) and attempts at trapping radical intermediates during turnover have failed because the 3'-hydrogen abstraction is not a rate-limiting step (7). However, in earlier studies involving suicidal substrate analogues, nucleotide localized radicals have been identified (8–10). In addition, a strongly cobalt-coupled thyl radical has been identified in the cobalamin-dependent class II RNR (2).

We recently trapped two kinetically coupled transient radicals in a reaction involving physiological substrate and the active site mutant R1 protein E441Q, which were formed at the expense of the stable tyrosyl radical of protein R2 (11). One intriguing result was that the second intermediate had EPR characteristics compatible with it being a substrate radical intermediate. We therefore postulated that the suicidal E441Q reaction mimicked the first few steps of the wild-type ribonucleotide reductase mechanism. Thus, the advantage of using the E441Q mutant is that the reaction is sufficiently slow to allow trapping and characterization of the intermediates. In this study, we have used the isotope labeling technique to identify the first transient radical as a cysteinyl radical in protein R1 and the second, as a carbon-centered substrate radical.

EXPERIMENTAL PROCEDURES

Materials—Isotopically labeled D-cysteine (3,3',3'-d4, 98%) and cytidine-5'-triphosphate (U-13C9, U-15N3) were prepared by Cambridge Isotope Laboratories. CDP, myosin, benzyl-DEAE-cellulose and triethylamine were purchased from Sigma, and dTTP (100 mM, pH 7.5) was from Pharmacia Biotech. Triethylamine boric acid buffer was prepared by titrating 1 M triethylamine with gaseous CO2 to pH 8.0.

Preparation of the mutant R1 protein E441Q was described previously (11).

Bacterial Strain—A cysteine auxotroph, E. coli AT2427 (λ-, e14, cyA43, relA1, spoT1, thi-1, creC510, Hfr) (12, 13), was obtained from E. coli Genetic Stock Center, Yale University, and transformed with plasmids pGP1-2 and pTB1/E441Q (11, 14).

Expression and Preparation of [β,β'-2H2]Cysteine-labeled R1 E441Q—E. coli AT2427 containing the plasmids pGP1-2 and pTB1/E441Q was grown at 30 °C in a 10-ml preculture of minimal salts medium containing 0.10 the concentration of trace metals (15) and 1.7 mM sodium citrate and supplemented with [β,β'-2H2]cysteine (80 μM) as...
the cysteine source, thiaminium dichloride (5.9 μM), glucose (0.4%), kanamycin (50 μg/ml), and carbenicillin (50 μg/ml). 0.4–0.65 ml of preculture was used to inoculate 400 ml of the same medium containing [β,β-3H]cysteine (160 μM). Five flasks containing 1.25 liters of the same medium were inoculated with this culture and grown at 30 °C with vigorous shaking (280 rpm). When the cultures reached exponential phase (A_{650} = 0.9), the temperature was raised to 42 °C to induce the overproduction of the cloned R1 gene. When the cultures reached stationary phase (A_{650} = 1.9 after 3 h of induction) the cells were quickly chilled on ice and harvested by centrifugation. Pellets were frozen on dry ice and stored at −80 °C. Preparation of the deuterium-labeled E441Q protein was as described for unlabeled protein (11). Purification was monitored by SDS-polyacrylamide gel electrophoresis with Coomassie Blue and silver staining.

**Protein Determination**—Protein concentrations were determined using the absorbance at 280 nm minus the absorbance at 310 nm and the extinction coefficients (ε_{280-310}: 180,000 M^{-1} cm^{-1} for protein R1 and 120,000 M^{-1} cm^{-1} for protein R2). The stained SDS-polyacrylamide gel electrophoresis gels were scanned in a Molecular Dynamics Inc. laser densitometer to calculate the purity of the protein preparations. Preparation of 13C-Labeled CDP—The 13C-labeled CDP was obtained from labeled CTP by incubation with myosin to hydrolyze the remaining triethylamine was removed by washing with methanol and evaporation for three cycles. The CDP was dissolved in 20 mM Tris-HCl, pH 7.6.

**EPR Samples and Measurements**—The reactions were performed at 25 °C by manual mixing of equal volumes of the protein solution (150 μM R1, 160 μM R2) in 50 mM Tris-HCl, pH 7.6, 15 mM Mg(CH_{3}COO)_{2}, 0.2 mM EDTA, 5 mM dithiothreitol) and the substrate solution (3.34 mM CDP in the same buffer). Samples containing protein solution and buffer without substrate were used to evaluate the initial concentration of tyrosyl radical. 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 were 2 s or longer. For anaerobic experiment, distilled water, 10 times concentrated buffer, and concentrated substrate in an anaerobic Eppendorf tube and flushing with argon in three separate tubes for 1 h. The protein solution (as above) was prepared by anaerobic mixing of water, concentrated buffer, and protein solutions in an anaerobic Eppendorf tube and flushing with argon for another 30 min, and then transferring to an anaerobic EPR tube. The substrate solution (as above) was prepared by anaerobic mixing of water, concentrated buffer, and concentrated substrate in an anaerobic Eppendorf tube and flushing with argon for another 30 min. The reaction was started as described above by addition of the anaerobic substrate solution to the anaerobic protein solution in the EPR tube using a gas-tight syringe.

**EPR Simulations**—The components of the g-tensor and the hyperfine tensors for two protons were determined with the help of a computer-simulated spectrum, according to Bernhard and Fousse (18).

**RESULTS**

**Direct Evidence for a Transient Cysteinyl Radical in the Reaction with R1 E441Q, Wild-type R2, and CDP**—In an earlier study, we reported that a transient mechanism-based radical was observable between 0.5 and 90 s in incubations involving the active site mutant R1 E441Q (11). Here we present the EPR characteristics of this early intermediate. After 10-s reaction with CDP at 25 °C the composite spectrum shown in Fig. 1b appears. Compared with the starting spectrum of the stable tyrosyl radical in Fig. 1a, the line shape in Fig. 1b has changed, and we observe a broadening in the low field end of the tyrosyl radical spectrum; additionally, the high field part is lowered compared with the spectrum in Fig. 1a. Subtraction of an appropriate portion of tyrosyl radical (11) gives the intermediate(s) shown in Fig. 2a. It can be described as a singlet with g = 2.01 and possibly an additional signal in the high field region. In Fig. 2a there is also a small contribution in the high field region from the triplet EPR spectrum of the next intermediate (see below).

To deduce the origin of this early singlet EPR signal (Fig. 2a) we have used isotopically labeled protein R1 and substrate. A change in the EPR spectrum of the transient signal is observed when incubations were performed with an E441Q protein labeled with β-deuterated cysteine (Figs. 1d and 2c). The line width of the low field part of the spectrum is narrowed from 2.7 to 1.9 mT at half-height. The features at high field also seem to have been sharpened by the deuteration, but this could also be the result of the spectrum of the next intermediate overlapping less with the singlet in this case. A second separately deuterium-labeled and purified sample gave an identical spectrum. An anaerobic incubation (Fig. 2d) also gave identical results to the aerobic incubations (Fig. 2a), apart from an insignificant difference in the hyperfine structure at high field. The latter most probably relates to a slightly higher relative contribution of the subsequent triplet signal in this particular experiment. In addition, the resulting spectra after incubation of E441Q and substrate uniformly labeled with 13C and 15N (Figs. 1c and 2b) are essentially identical to spectra obtained with unlabeled substrate (Figs. 1b and 2a). These data demonstrate that the early transient oxidized intermediate observed is a nonoxidized cysteinyl radical in protein R1.

**Direct Evidence for a Transient Carbon-centered Substrate Radical in the Reaction with R1 E441Q, Wild-type R2, and CDP**—The previous study also suggested that the early singlet EPR signal was followed by an intermediate triplet signal with EPR characteristics compatible with a substrate radical (11). In this study we show that incubation with uniformly 13C- and 15N-labeled CDP gives rise to a collapse of the 1:2:1 triplet EPR spectrum of the unlabeled second intermediate to a broad singlet spectrum (Figs. 3b and 4A, spectrum a), demonstrating that this is a carbon-centered radical. A nitrogen-centered radical is excluded as it would be a 1:1:1 triplet in the unlabeled sample that would collapse to a doublet in an 15N-labeled sample. Simulations of the EPR spectrum of the substrate radical gives an almost perfect fit with hyperfine couplings to two I = 1/2 species, one almost isotropic and one slightly anisotropic. The experimental spectrum and the simulation are...
obtained after 3 min of incubation with uniformly 13C-labeled CDP reaction run as in a.

resulting spectrum after an anaerobic reaction run as in a. The long arrow indicates $g = 2.01$ and the short arrow $g = 2.03$.

shown in Fig. 4B, and the resulting tensors for the $g$ value are (2.0066, 2.0062, 2.0024), and interacting protons are in mT $a_{H1}$ (0.78, 0.88, 0.88) and $a_{H2}$ (1.06, 1.47, 0.88). As a control we show that incubation of the cysteine-deuterated E441Q protein with normal 13C-containing CDP did not perturb the EPR spectrum of the late transient intermediate (Figs. 3, c and d, and 4A, spectra b and c). These data demonstrate that the second transient intermediate is a carbon-centered substrate radical intermediate with hyperfine couplings to two almost identical protons.

The Cysteinyl Radical and the Substrate Radical Are Consecutive Intermediates in the Reaction—The kinetics of the reaction and the relative yields of transient radicals are within experimental error the same in the presence of R1 E441Q labeled with $\beta$-deuterated cysteine, 13C-labeled substrate, or with unlabeled substrates. In addition, the kinetics and yields of transient radicals obtained in the current study are comparable with the kinetic data obtained in the previous study (11). This underscores the generality of the reaction intermediates observed, where the formation and decay of the cysteinyl and substrate radicals fit the kinetic model of two consecutive intermediates with formation rates of $\approx 1.2$ and 0.03 s$^{-1}$, respectively (11).

The Cysteinyl Radical of R1 Has a Saturation Behavior in between That of the Substrate Radical and the Tyrosyl Radical of Protein R2—The microwave power at half-saturation ($P_{1/2}$) of the cysteinyl radical measured at 77 K is 1.1 mW (Fig. 5). The corresponding $P_{1/2}$ values for the substrate radical and the stable tyrosyl radical of protein R2 are 72 $\mu$W and 12.9 mW, respectively (Fig. 5). The $P_{1/2}$ value of the substrate radical is as expected for an isolated carbon-centered free radical species, whereas the $P_{1/2}$ value of the tyrosyl radical reflects its strong magnetic interaction with the adjacent diiron site (17). Interestingly, the higher $P_{1/2}$ value of the cysteinyl radical as compared with that of the substrate radical implies that the cysteinyl radical is in weak magnetic interaction with a fast relaxing species. The only species that could give relaxation in our incubation system is the diiron site of R2, which at a distance of 35–40 Å is in hydrogen-bonded connection with Cys439 of protein R1. However, it cannot be excluded that a sulfur-centered radical, due to its different electron distribution, might, by itself, display a more metal-like saturation behavior than an isolated carbon-centered radical. We have not been able to find published $P_{1/2}$ values for sulfur radicals, but in a report by Kertesz et al. (19) it is stated that no saturation was observed at room temperature even at 200 mW. Generally EPR signals of sulfur radicals are measured at microwave powers of 1–2 mW at 77 K.

**DISCUSSION**

In a recent study on class Ia RNR of *E. coli* we trapped two consecutive mechanism-based radical intermediates in a reaction involving physiological substrate, the engineered R1 protein E441Q, and wild-type protein R2 (11). In the current study we have used isotope labeling of the mutant R1 protein E441Q...

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**Fig. 2.** EPR spectra of the transient cysteinyl radical in the reaction of E441Q R1 with wild-type R2 and CDP. Spectra were obtained after subtraction of a fraction of the spectrum in Fig. 1a. Resulting spectra for 10-s incubation (a), with unlabeled substrate and proteins (b), with uniformly 13C-labeled CDP, and with [β,β-2H]cysteine-labeled R1 E441Q (c). A fraction of doublet tyrosyl radical, 45% of the total doublet integral of the composite spectrum after 10 s of incubation, has been subtracted. d, resulting spectrum after an anaerobic reaction run as in a. The long arrow indicates $g = 2.01$ and the short arrow $g = 2.03$.

**Fig. 3.** X-band EPR spectra of radical signals obtained after 3-min incubation at 25 °C of E441Q R1 with wild-type R2 and CDP, a, starting spectrum before addition of substrate, b–d, spectra obtained after 3 min of incubation with uniformly 13C-labeled CDP, with [β,β-2H]cysteine-labeled R1 E441Q (c) and with unlabeled substrate and proteins (d). EPR conditions were: temperature, 77 K; six scans; modulation amplitude, 0.16 mT; microwave power, 18.5 $\mu$W; receiver gain, 8 × 10$	extsuperscript{5}$.

**Fig. 4.** A. X-band EPR spectra of the transient substrate radical in the reaction of E441Q R1 with wild-type R2 and CDP. Spectra were obtained after subtraction of a fraction of tyrosyl radical in the control corresponding to 5.2 $\mu$W from spectra b–d in Fig. 3. The following intermediates were observed with uniformly 13C-labeled CDP (a), with [β,β-2H]cysteine-labeled R1 E441Q (b), and with unlabeled substrate and proteins (c). The resulting substrate radical was determined to 7.2, 7.2, and 6.5 $\mu$W in traces a–c, respectively. The arrow indicates the $g$ value of 2.005. B, experimental and simulated spectra of the substrate radical after 2-min incubation with CDP. In the experimental spectrum the spectrum of the remaining Tyr122 radical has been subtracted. EPR conditions were: temperature, 77 K; 18 scans; modulation amplitude, 0.16 mT; microwave power, 18.5 $\mu$W; receiver gain, 8 × 10$	extsuperscript{5}$.

To reduce the number of data points to fit in the simulation, the spectrum consists of 512 point over 10 mT. Thin line, simulated spectrum; thick line, the experimental spectrum. The arrow indicates the $g$ value of 2.005.
to show that the early transient radical with an EPR singlet at $g = 2.01$ is localized to a cysteine residue in protein R1. It is observable in the time interval $-0.5$ to $90$ $s$ and kinetically coupled to the decay of the tyrosyl radical of protein R2 and the formation of the second transient radical localized to the substrate nucleotide (Fig. 6). The microwave saturation behavior of the cysteinyl radical shows that it is far away from the diiron site of R2. Yet, its $P_0$ value is slightly higher than expected for a carbon-centered free radical (17), which may suggest that it is weakly connected to the diiron site of R2. A plausible candidate is Cys439 at the active site of R1. Cys439 has been postulated to harbor a transient radical during catalysis, which is thought to abstract the 3'-hydrogen atom of the substrate nucleotide (1, 20).

Few cysteine-related radicals in proteins have been reported in the literature. In pyruvate formate lyase a disulfide radical is formed when the enzyme is inactivated with mercuricpyruvate, and a sulfinyl (and a peroxyl) radical is formed when the enzyme is inactivated with mercaptopyruvate nucleotide (1, 20). Both these radicals display hyperfine coupling to one or two $\beta$-protons of the cysteine residue. A transient radical with $g_1 = 2.11$ and $g_e = 2.03$ has been reported when aconitate is reacted with nitric oxide, and it has been assigned to a thyl radical by comparison with values reported for chemically induced species (23, 24). In class Ia RNR from \textit{E. coli}, a disulfide radical with $g_e = 2.062$, $g_d = 2.0265$, $g_1 = 2.0091$ has been observed when wild-type protein was incubated with 2'-deoxy-2'-mercaptothymidine 5'-diphosphate (25). This radical shows hyperfine coupling to one $\beta$-methylene proton of cysteine. Finally, in class II RNR of \textit{Lactobacillus leichmannii} a strongly cotryptic thyl radical has been described (2). As the thyl part of this radical is at $g = 2.12$, its EPR features are clearly different from those of the mechanism-based cysteinyl radical described here.

In protein-free systems cysteine-derived radicals have mostly been generated after $\gamma$-irradiation of frozen cysteine solutions; \textit{i.e.} the primary radical product is the result of a one electron addition. The reported radicals most similar to our observed cysteinyl radical are (i) the thyl radical produced after chemical oxidation of hydroxythiol with Co$^{4+}$ in a contin-

**Fig. 6. Comparison between the proposed reaction scheme for E441Q R1 incubated with CDP and wild-type R2 and the wild-type ribonucleotide reductase mechanism.** Three different radical species and the end product have been identified in the kinetically coupled reaction sequence with E441Q (this study and Ref. 11). The exact location of the cysteinyl radical in R1 and the carbon-centered substrate radical is tentative. The reaction rates ($k_1$ to $k_3$) relate to the E441Q reaction and are from Persson \textit{et al.} (11); the overall $k_{cat}$ value of the wild-type reaction is also shown.

The structureless feature of the cysteinyl EPR signal resulting after subtraction of the stable Y122$'$ makes it difficult to positively identify the type of radical observed. At times longer than $10$ $s$, a substantial amount of substrate radical has formed and has to be subtracted to give a "pure" thyl radical spectrum. At shorter times yet another species is observed, with features observable in the high field part of Fig. 2. This species is under investigation. One possibility would be that the low field feature at $g \approx 2.03$ comes from a small fraction of thyl radicals that have reacted with oxygen, which would account for the poor resolution of the spectrum. However, we consider this unlikely, since the $g \approx 2.03$ feature is also present in the spectrum of an anaerobically reacted sample (Fig. 2d).

The second transient radical intermediate with a characteristic triplet EPR signal was in our earlier study tentatively assigned to a substrate radical (11). In this study we directly corroborate this assignment; use of a uniformly $^{13}$C-labeled CDP substrate results in an unquestionable change of the triplet into a broad singlet. Its microwave saturation behavior is that of a carbon-centered free radical species. As schematically shown in Fig. 6, the substrate radical is a kinetically coupled intermediate between the decay of the transient cysteinyl radical in R1 and the formation of a nucleotidederived furanone species (11), generally observed as end product in suicidal $k_{cat}$ inhibitor reactions with different RNRs (27).

Simulation of the triplet EPR signal of the substrate radical identifies coupling to two protons: one which is almost completely isotropic ($-0.85$ $mT$) and one with a somewhat larger anisotropic character ($H_{iso} = 1.14$ $mT$). A recent structural study of a GDP substrate bound to the active site of the R1 protein suggests that the bound nucleotide adopts either 2'-or 3'-endo puckering (28). The simulated hyperfine couplings would be compatible with a transient 3' radical species sensing the 2'- and 4'-protons. The structureless singlet EPR spectrum obtained with the uniformly $^{13}$C-labeled substrate does not give any additional information as to the spin density distribution of the unpaired electron in the substrate, and such studies have to use site-specifically labeled substrate.

A few nucleotide radicals observed in reactions with inhibit-
ing nucleotide analogues have been reported recently. These include (E/Z)-2’-fluoromethylene-2’-deoxycytidine 5’-diphosphate and gemcitabine (2’-deoxy-2’,2’-difluorocytidine) 5’-diphosphate (8–10). The 2’-fluoromethylene nucleotide intermediates have g ~ 2.003, and both have hyperfine splittings of ~1.5 mT (deduced from Fig. 3 (8) after field correction, and Fig. 1 (9), respectively). The 2’-difluoro nucleotide intermediate has a g value that is slightly higher than that of the tyrosyl radical (g = 2.005) and a hyperfine splitting of 1.0–1.2 mT (deduced from Fig. 5 (10). In addition, only the reported P$_{1/2}$ value for the (Z)-2’-fluoromethylene nucleotide intermediate is as expected for a carbon-centered free radical species. Thus, neither of these three radicals are identical to the nucleotide radical observed by us as a kinetically coupled intermediate in a reaction with a physiological substrate.

Earlier studies with mutant R1 proteins altered in an active site residue showed substrate-dependent cleavage of the polypeptide chain at the engineered site (29). In the studies reported here, several kinetically competent intermediate radicals are formed, but no truncation of the mutant R1 polypeptide chain occurs, suggesting that the reaction sequence and the intermediates observed are closer to a physiological situation. As indicated in Fig. 6 the cysteinyl and the nucleotide radical intermediate may plausibly be equivalent to two intermediates postulated for the wild-type reaction. To determine the true nature of these intermediates further studies with specifically isotopically labeled substrates and protein components, as well as additional engineering or modification of the mutant R1 E441Q protein are needed.

To conclude, this is the first report of a cysteinyl free radical in RNR that is a kinetically coupled precursor of an identified substrate radical and that has properties compatible with the postulated thyl radical at Cys$_{439}$ generally considered to initiate catalysis. The nature of this cysteinyl radical has to our knowledge not previously been observed in a protein. Further characterization with mutated proteins and isotope labeling is being pursued to further delineate the reaction mechanism, including the intriguing radical transfer reactions of the RNR enzyme.

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