The Stable Tyrosyl Radical in Mouse Ribonucleotide Reductase Is Not Essential for Enzymatic Activity

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Received July 5, 1994

Mouse ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides to their corresponding deoxyribonucleotides, providing the precursors for *de novo* DNA synthesis.[1,2] In common with other eucaryotic and some bacterial RRs, the mouse enzyme is comprised of two homodimeric subunits, R1 and R2. The mechanism of action is thought to involve a long-range electron transfer between a stable tyrosyl radical (Y177 in mouse, Y122 in *Escherichia coli)* in the R2 subunit and the substrate bound to R1. Evidence for the central role of Y122 in the *Escherichia coli* enzyme was provided by the low activity observed for the *E. coli* Y122F-R2 variant (1–2% of wild-type).[3] It was unclear from this experiment whether this small amount of activity reflected the presence of a small amount of chromosomally encoded wild-type R2 contaminating the Y122F-R2 preparation or whether it was due to an intrinsic activity of the Y122F-R2 variant. In order to allow more definitive analysis the central role of the tyrosyl residue in catalysis, the mouse Y177F-R2 variant was generated in an *E. coli* expression system.[4] The structural integrity of the mutant was confirmed by the close similarity of its CD spectrum and stoichiometry of iron binding (Y177F, 3.3/subunit; WT, 3.2/subunit), as compared to wild-type R2. We find that combining mouse Y177F-R2 with saturating amounts of recombinant mouse R1[5] generates an enzyme having 0.5% the RR activity of the wild-type enzyme.

As summarized in Table 1, the inhibition of Y177F-R2-dependent activity (0.8 nmol/min/mg) displays properties characteristic of RRs in general and of mammalian RR in particular. It is inhibited by low concentrations of the negative allosteric effector, dATP (IC_{50}, 20 μM), in the presence of the positive effector, ATP.[6] Hydroxyurea, which destroys both the tyrosyl radical and the iron center in mammalian R2,[7,8] inhibits the activity of Y177F-R2 as well (IC_{50}, 210 μM).

Peptides corresponding to the C-termini of the R2 subunits are known to compete with R2 for binding to R1, thereby inhibiting RR activity.[9,10] This inhibition is specific, reflecting the difference in C-terminal sequences of R2s from different organisms. Recently we demonstrated that mammalian RR is strongly inhibited by *N*-AcFTLDADF, corresponding to the C-terminus of mouse R2, but only weakly inhibited by the similar peptide *N*-AcFTLDADL.[11] That the enzymatic activity we attribute to Y177F-R2 shows this same specificity toward peptide inhibition is strong evidence that it does not result from contamination with host RR present in the recombinant R1 or Y177F-R2 preparations.

Two further results demonstrate that the mammalian RR activity we measure for Y177F-R2 is not due to a small amount of contaminating wild-type RR. First, no tyrosyl radical was detected in a 270 μM solution of Y177F-R2 using EPR conditions capable of detecting as little as 0.5 μM wild-type R2 tyrosyl radical (Figure 1). Approximately 1.5 μM wild-type R2 would have been required to account for the observed activity. Second, PCR analysis of DNA extracted from *E. coli* expressing Y177R-R2, using wild-type primers, failed to detect a PCR product, indicating the absence of a gene encoding wild-type R2 (Figure 2). The limit of detection for this analysis was determined to be 1 part in 1000, much lower than the 1 part in 200 that would be needed to account for the observed RR activity.

The commonly accepted mechanism of catalysis of RR begins with the assembly of the diiron center on the R2 subunit. Two ferric ions and the stable tyrosyl radical are produced as a result

Table 1. Y177F-R2 Activity[a]

	activity (nmol/min/mg)
R1+Y177F-R2	0.8
R1+R2	150.0
R1	ND
R2	ND
Y177F-R2	ND
N-AcFTLDADF (μM)	
30	0.7
100	0.3
300	0.1
1000	ND
N-AcFTLDADL (μM)	
30	0.7
100	0.8
300	0.8
1000	0.9
dATP (μM)	
1	0.9
3	0.8
10	0.7
30	0.4
100	0.2
300	0.1
1000	ND
Hydroxyurea (μM)	
3	1.0
10	1.1
30	0.8
100	0.7
300	0.4
1000	0.2
3000	0.1

[a] A typical assay mixture contained (100 μL): 100 μg of BSA, 60 mM HEPES (pH 7.6), 25 mM DTT, 0.05 mM $FeCl_3$, 8.75 mM NaF, 2.7 mM $Mg(OAc)_2$, 3 mM ATP, 35 nM [^{3}H]CDP (18 Ci/mmol), and 30 μM CDP. When indicated, increasing concentrations of either dATP, *N*-AcFTLDADF, *N*-AcFTLDADL, or hydroxyurea were added. Reaction was initiated by addition of R1 (molecular weight 180 000; 4.4 μM) and Y177F-R2 (molecular weight 90 000, 1.1 μM). Each protein (R1, 4.4 μM; R2, 8.4 μM; and Y177F-R2, 8.4 μM) was also tested alone to ensure that activity was dependent on subunit association. Incubation time, 30 min; 37 °C. The specific activity for the native system was measured with 10-fold less enzyme and an incubation time of 3 min. Reactions were quenched by immersion in boiling water (4 min). Conversion of CDP to dCDP was measured.[17] Each value is the average of two determinations: deviations ≤ 0.2. ND means not detectable.

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(1) Stubbe, J. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1990**, *63*, 349–419.
(2) Fontecave, M.; Nordlund, P.; Eklund, H.; Reichard, P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1992**, *65*, 147–183.
(3) Larsson, A.; Sjoberg, B. M. *EMBO J.* **1986**, *5*, 2037–2040.
(4) Mann, G. J.; Graslund, A.; Ochiai, E. I.; Ingemarson, R.; Thelander, L. *Biochemistry* **1991**, *30*, 1939–1947.
(5) Salem, J. S.; Scott, C. P.; Li, L. S.; Cooperman, B. S.; Rubin, H. *FEBS Lett.* **1993**, *323*, 93–95.
(6) Thelander, L.; Eriksson, S.; Akerman, M. *J. Biol. Chem* **1980**, *255*, 7426–7432.
(7) McClarty, G. A.; Chan, A. K.; Choy, B. K.; Wright, J. A. *J. Biol. Chem.* **1990**, *265*, 7539–7547.
(8) Nyholm, S.; Thelander, L.; Graslund, A. *Biochemistry* **1993**, *32*, 11569–11574.
(9) Cohen, E. A.; Gaudreau, P.; Brazeau, P.; Langelier, Y. *Nature* **1986**, *321*, 441–443.
(10) Dutia, B. M.; Frame, M. C.; Suback-Sharpe, J. H.; Clark, W. N.; Marsden, H. S.; *Nature* **1986**, *321*, 439–441.
(11) Fisher, A.; Yang, F.-D.; Rubin, H.; Cooperman, B. S. *J. Med. Chem.* **1993**, *36*, 3859–3862.

0002-7863/94/1516-9773$04.50/0

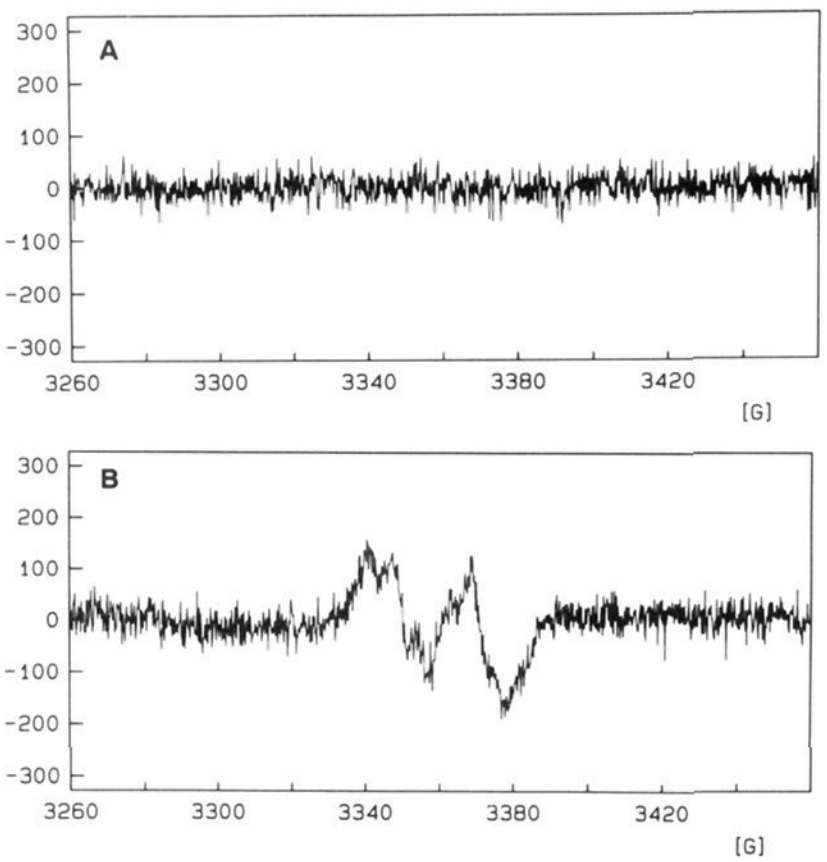

Figure 1. EPR spectra of Y177F-R2 and WT-R2 at nonsaturating microwave power conditions: (A) 270 μM Y177F-R2. (B) 0.5 μM WT-R2. Recorded at 30 K (Bruker 300E spectrometer). Microwave power, 0.5 mW; microwave frequency, 9.44 GHz; modulation amplitude, 4.0 G. Samples were dissolved in radical regenerating buffer [$Fe(NH_4)_2(SO_4)_2$, 10 mM; Na ascorbate, 100 mM; Tris·HCl (pH 7.6), 50 mM]. Ordinate: signal intensity (arbitrary units); abscissa: magnetic field (gauss).

of the four-electron reduction of O_2, with an external reductant supplying the fourth electron.[12] Because the tyrosyl radical in *E. coli* R2 is known to be buried 10 Å from the surface of the protein,[13] a long-range electron transfer is proposed in the generation of a substrate radical on the R1 subunit, a proposed intermediate in substrate reduction. Based on the specific activity of wild-type R2 (150 nmol/min/mg R2) and the stability of the Y177 radical in the assay medium ($t_{1/2}$ = 10 min),[14] approximately 100 turnovers of substrate are achieved per tyrosyl radical for wild-type RR.

Transient formation of other tyrosine and tryptophan radicals has been observed in studies of the Fe-center reaction using the Y122F-R2 protein from *E. coli*.[12,15] We presume that such radicals also form in mouse Y177F-R2 and hypothesize that they can substitute for Y177 in catalyzing substrate reduction. The

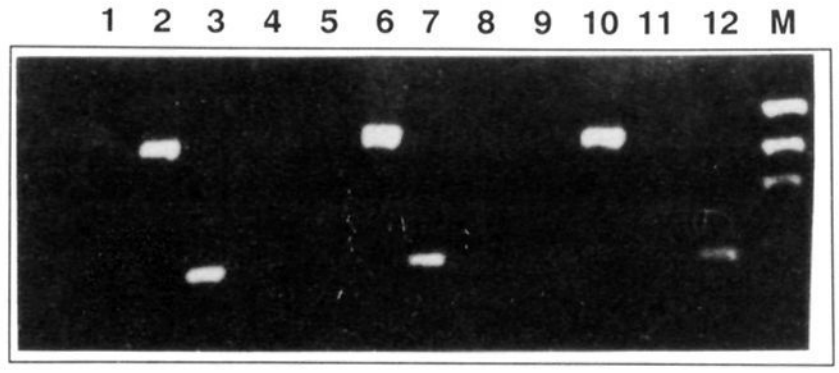

Figure 2. PCR amplification using Y177F-R2 and WT-R2 primers of DNA purified from Y177F-R2 expressing cells. DNA was isolated from BL21(DE3) cells expressing the recombinant protein used for this study (lanes 1–4) or from DH5a cells harboring either the Y177F- (lanes 5–8) or WT- (lanes 9–12) R2 gene. Plasmid DNA (10 ng) was amplified using single primer or primer pairs; N-term alone (lanes 1, 5, 9); N-term/C-term (lanes 2, 6, 10); N-term/Y177F-R2 (lanes 3, 7, 11) N-term/WT-R2 (lanes 4, 8, 12). Amplification was performed for 25 cycles (94 °C, 15 s; 50 °C, 20 s; 72 °C, 45 s). The detection limit for WT-R2 amplification was determined using dilutions of plasmid DNA amplified using N-term and WT-R2 primers as above. Primers used in this study; N-term, (5′-CCCCGCTAGCATGCTCTCCGTCCGCACC-3′); C-term, (5′-CCCCGCTAGCTTAGAAGTCAGCATCCA-3′); Y177F-R2-primer (5′-GTCAATAAGGAGACTGA-3′), WT-R2-primer (5′-GTCAATAAGGAGACTGT-3′). Lane M corresponds to an *Hae*III digest of Φx174 having fragment sizes (in bp): 1353, 1078, 872, and 603.

much lower activity measured for Y177F-R2 may reflect a less favorable electron transfer pathway from the residue substituting for Y177. Alternatively, or in addition, it may reflect lower stability of the substitute radical. For example, it might be that each turnover of substrate would require regeneration of the oxidized diiron center and the substitute radical, making the generation of such a radical the rate-determining step for catalysis.

Finally, it is worth noting that the diiron center of mouse R2 is much more labile than that of *E. coli* R2,[14,16] undergoing continual regeneration during *in situ* activity assays. Thus, we would predict that, except perhaps for the first turnover, the Y122F-R2 *E. coli* variant would have even lower specific activity than what we measure for mouse Y177F-R2 if regeneration of a substitute radical became rate-determining. We have measured 4.4 turnovers per Y177F-R2, though higher values may be obtainable.

Acknowledgment. This research was supported by Sterling Drug Corporation and Apollon Inc. We thank Alison Fisher for providing peptides, Charles Scott for providing recombinant R1, Christian Hamann for assistance expressing Y177F-R2, Nora Zuno for technical support, and Dan Robertson for assistance with EPR.

(12) Bollinger, J. M.; Edmonson, D. E.; Huynh, B. H.; Filley, J.; Norton, J. R.; Stubbe, J. *Science* **1991**, *253*, 292–298.
(13) Nordlund, P.; Sjoberg, B. M.; Eklund, H. *Nature* **1990**, *345*, 593–598.
(14) Thelander, L.; Graslund, A.; Thelander, M. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 859–865.
(15) Sahlin, M.; Lassmann, G.; Patsch, S.; Slaby, A.; Sjoberg, B. M.; Graslund, A. *J. Biol. Chem.* **1994**, *269*, 11699–11702.
(16) Furukawa, T.; Naitoh, Y.; Kohno, H.; Tokunaga, R.; Taketani, S. *Life Sci.* **1992**, *50*, 2059–2065.
(17) Moore, E. C.; Peterson, D.; Yang, L. Y.; Yeung, C. Y.; Neff, N. F. *Biochemistry* **1974**, *13*, 2904–2907.