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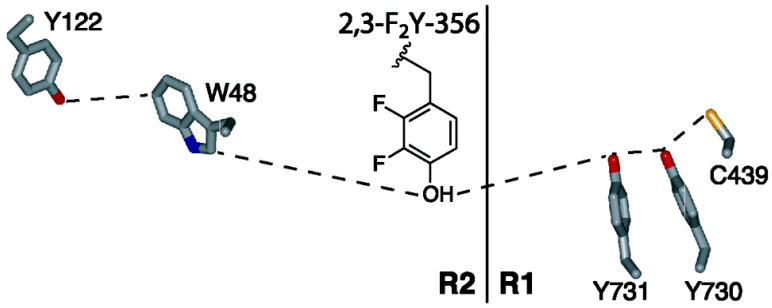

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2,3-Difluorotyrosine at Position 356 of Ribonucleotide Reductase R2: A Probe of Long-Range Proton-Coupled Electron Transfer

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Ribonucleotide reductases (RNR) catalyze the conversion of nucleotides to deoxynucleotides, an essential step in DNA replication and repair in all organisms.[1] The *Escherichia coli* (*E. coli*) RNR is composed of two homodimeric subunits, R1 and R2, that are proposed to form a 1:1 active complex. R1 binds the substrates and effectors that control turnover and specificity, while R2 contains the essential di-iron tyrosyl radical (Y•) cofactor. While the unusual radical mechanism of nucleotide reduction has been extensively studied and is moderately well understood,[2] the mechanism of radical initiation, how a Y• on the R2 subunit generates a transient thiyl radical (S•) on the R1 subunit, remains unresolved. A docking model of the R1 and R2 subunits based on shape complementarity suggests that the initiation occurs over 35 Å (Figure 1).[3] Marcus theory predicts that electron tunneling over such a distance is extremely slow ($\sim 6 \times 10^{-6}$ s^{-1}),[4,5] requiring amino acid radical intermediates to account for the observed rate constant of 10 s^{-1} for nucleotide reduction. The proposed pathway involves several tyrosines, a tryptophan, and a cysteine residue. The radical initiation mechanism is thought to involve proton-coupled electron transfer (PCET),[5a,6] because the redox properties of these amino acids can be modulated by their protonation states[7] and because tyrosine is unable to oxidize cysteine (redox potentials at pH 7.0 of 0.77 and 1.33 V vs NHE,[8] respectively). Although mutagenesis studies of the residues in this pathway and their conservation in all class I RNRs establish their importance in nucleotide reduction, evidence to support a specific pathway for radical initiation has been absent. We now report a semisynthesis of R2 using intein technology with an unnatural amino acid (X) in position 356. Studies in which X is 2,3-difluorotyrosine (F_2Y) have allowed us to test the importance of direct or stepwise hydrogen atom transfers between W48 of R2 and C439 of R1.[6] Radical transfer between Y122• and W48 is proposed to involve a tunneling mechanism.[5a]

The structure of *E. coli* R2 reveals the first 340 of its 375 residues. The remaining residues of the C-terminal tail are thermally disordered.[9] Y356, which is essential for nucleotide reduction,[10] resides in this disordered region and is proposed to be on the radical initiation pathway.[11] The flexibility of the C-terminus suggested that R2 could be made semisynthetically using molecular biology for the majority of the protein and a peptide synthesizer to generate the C-terminus.[12] The successful experimental approach is shown in Scheme 1. Residues 1−353 were fused to the *Vma*I intein and a chitin-binding domain (CBD). The desired protein could be isolated with a chitin affinity column in 100 mg quantities.[13,14]

The isolated protein was ligated with peptide C_{354}SXLVGQID-SEVDTDDLSNQFL$_{375}$, corresponding to residues 354−375 of the C-terminal tail of R2.[15] In this work, Y356(X) was substituted with F_2Y, and S354 in wild-type-R2 (wt-R2) was replaced with a cysteine, which is required for intein ligation. To enhance the rate of ligation of the peptide with the thioester of R2$_{(1-353)}$ relative to the hydrolysis of this thioester, V353 was also replaced with an alanine or a glycine. The ligation resulted in a mixture of truncated homodimers, heterodimers, and the desired full length R2. Fortunately, the charge on the C-terminus of R2 was sufficient to allow separation of these different R2 forms by anion exchange chromatography. The results (A) and the ESI−MS (B) of the desired R2 are shown in Figure 2.

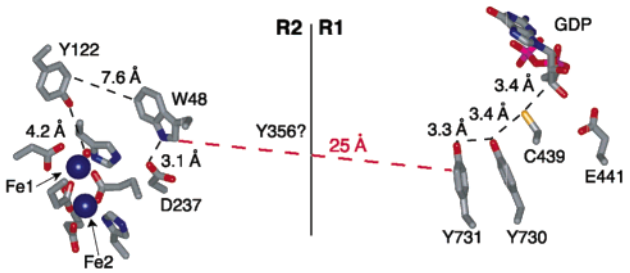
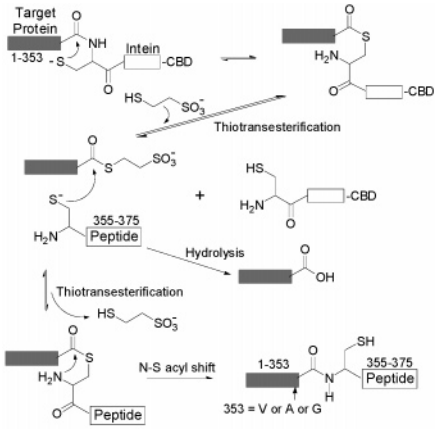


Figure 1. Proposed PCET pathway and distances based on crystal structures of R1 and R2.[3] The distance in red is based on the docking model.

Scheme 1. Intein Ligation



R2 has a di-iron Y• cofactor that must be assembled to be active. The reconstitution of apo-R2 was performed in a manner similar to our previously published procedures.[16] With both mutant and wt-V353G,S354C-R2, 0.6−0.7 Y•s per R2 were obtained.

The mechanism for radical initiation in class I RNRs has been proposed to involve a number of direct hydrogen atom transfer steps between W48 in R2 and C439 in R1.[6] The F_2Y-R2 was designed to test this mechanism directly. The pK_a of *N*-acetyl-2,3-difluorotyrosinamide is 7.8, and its redox potential is very similar to that of *N*-acetyltyrosinamide (Figure 3). We have shown that the pK_a of 3-nitrotyrosine (3-NO_2Y) in position 356 of R2 is not significantly perturbed when R2 is complexed with R1 in the presence of effectors (TTP, dGTP) and substrates (CDP, ADP).[13] If we assume that pK_a and redox potential of F_2Ys are similarly unperturbed by the protein environment, then the proposed hydrogen

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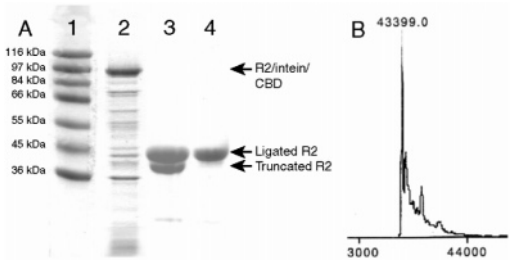

Figure 2. Purification of R2. (A) SDS-PAGE: lane 1, molecular weight markers; lane 2, crude lysate, showing expression of V353G-R2(1−353)/intein/CBD at 91.7 kDa; lane 3, 4 ug of ligation mixture, showing 70:30 full-length ligated R2 and truncated R2; lane 4, 2 ug of anion exchange purified ligated full length R2. (B) ESI−MS of purified F_2Y-R2. Calculated mass is 43 396 Da, and observed mass is 43 399 Da.

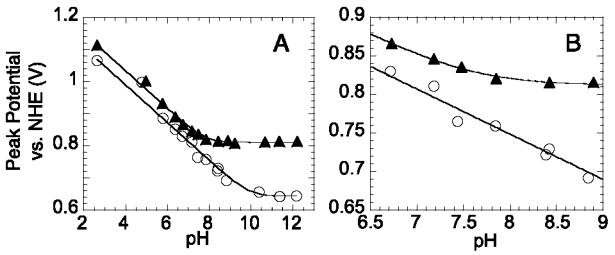

Figure 3. Differential-pulse voltammetry[7] over a wide pH range (A) and the pH range under which R2 is stable (B) of *N*-acetyl-2,3-difluorotyrosinamide (▲) and *N*-acetyltyrosinamide (○).

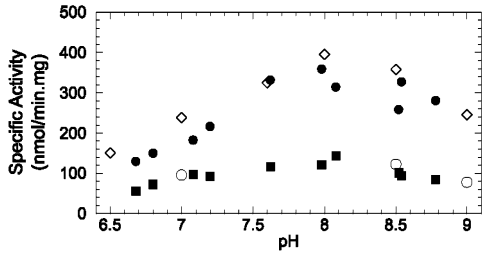

Figure 4. pH rate profile for wt-V353G,S354C-R2 (● and ◇) and V353G,-S354C,F_2Y-R2 (■ and ○). The specific activities of ligated wt-R2 and that prepared by site-directed mutagenesis are identical when corrected for Y• radical content.

abstraction mechanism predicts that nucleotide reduction should occur when the F_2Y is protonated. However, when F_2Y is deprotonated, deoxynucleotide reduction should not occur.

A pH rate profile was therefore generated for wt-V353G,S354C-R2 and V353G,S354C,F_2Y-R2, both made by the intein procedure, and the results are shown in Figure 4.

The pH profiles are remarkably similar, although the activity of the F_2Y-R2 is a factor of 2−3 less than that of wt-V353G,S354C-R2 throughout the entire pH profile.

These results thus suggest that a proton can be removed from the putative PCET pathway with no detrimental effect on nucleotide reduction. This finding is consistent with the current R1R2 docking model, where W48 on R2 and Y731 on R1 are 25 Å apart. Unless the proteins undergo substantial conformational changes that shorten this distance, then hydrogen transfers between W48 and Y731 are highly unlikely.

Finally, it is also chemically reasonable that the deoxynucleotide formation could occur after deprotonation as the phenolate of F_2Y is more readily oxidized than the corresponding phenol. Generation of R2, in which X is tri- or tetrafluorotyrosine, might provide the correct balance between redox potential and protonation state to demonstrate a pH-dependent switch for deoxynucleotide formation. The stage is now set to examine radical initiation of nucleotide reduction in class I RNRs.

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Supporting Information Available: Differential-pulse voltammetry and radioactive activity assay method (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) From 10 L of LB-media, we obtain 20 g of cell paste, containing approximately 200 mg of the R2/intein/CBD construct. Given the 50% intein cleavage efficiency and the 70% ligation efficiency, we typically obtain ~50 mg of purified full-length homodimeric protein. The detailed ligation and purification procedure will be found in ref 13.
(15) Solid-phase peptide synthesis was performed with Fmoc protected amino acids and *N*-((dimethylamino)-1*H*-1,2,3-triazolo(4,5-*b*)pyridin-1-ylmethylene)-*N*-methylmethananaminium hexafluorophosphate *N*-oxide (HATU) coupling. Cysteine was protected with a *t*Buthio, and residues D362 and S363 were replaced with a Fmoc-Asp(OtBu)-Ser(psiMe,Mepro)-OH dipeptide, which prevented extensive side reactions associated with cyclization and ring opening of aspartates. The synthesis was performed with standard procedures and will be found in ref 13.
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