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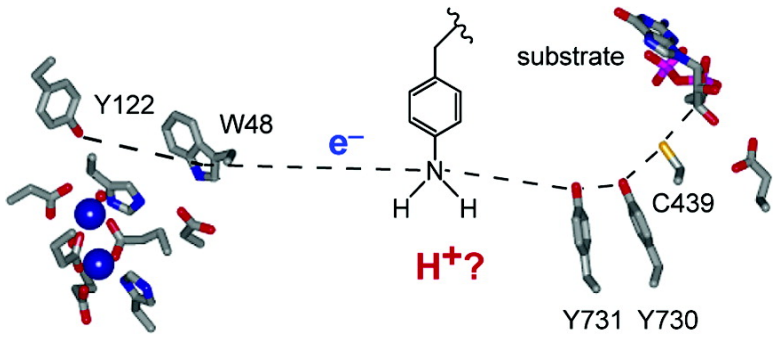

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Site-Specific Replacement of a Conserved Tyrosine in Ribonucleotide Reductase with an Aniline Amino Acid: A Mechanistic Probe for a Redox-Active Tyrosine

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Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides in all organisms, supplying a balanced pool of deoxynucleoside triphosphates required for DNA replication and repair.[1] The mechanism of nucleotide reduction involves complex radical chemistry and requires a transient protein-based thiyl radical (S•) to initiate the first step of catalysis.[2] Surprisingly, RNRs are found to contain structurally diverse metallo-cofactors, whose purpose is to generate the thiyl radical, and are thus grouped into three major classes. The class I RNR utilizes a diiron-tyrosyl (Y•) radical cluster, the class II RNR contains an adenosylcobalamin cofactor, while the class III RNR uses a glycyl radical generated by a [FeS]/S-adenosylmethionine activase.[1] The cofactors of the class II and III enzymes are found within 5−7 Å of the essential cysteine (C439, *Escherichia coli* numbering) and function by direct hydrogen abstraction from this residue.[3] However, radical initiation in the class I RNR is a much more complex problem,[4] as the tyrosyl radical cofactor is located on a second subunit and is thought to be separated from the active-site cysteine by 35 Å.[5]

The class I RNR from *E. coli*, which has long served as a model for the mammalian enzyme, is proposed to exist in a 1:1 complex of two homodimeric subunits, R1 and R2. R1 houses the conserved active site for nucleotide reduction and the binding sites for allosteric effectors, while R2 contains the diiron-Y122• cofactor. A docking model of the individual crystal structures of R1 and R2 provides the proposed distance of 35 Å between active sites and also places a pathway of absolutely conserved aromatic amino acids between Y122• and C439 (Figure 1).[5] On the basis of the difference between the known rate of nucleotide reduction ($1-10\ s^{-1}$) and the calculated rate of electron transfer (ET) over this long distance ($10^{-4}-10^{-9}\ s^{-1}$),[6] we arrive at a model of radical transfer that requires intermediates or radical hopping between aromatic amino acids. In vitro and in vivo mutagenesis studies have shown the aromatic residues of Figure 1 to be essential;[7] however, these types of studies have not been able to address the mechanism of radical transfer because nucleotide reduction activity is abolished rather than perturbed. Previous studies from our group have shown the importance of Y356 (in R2) and Y731 (in R1) in radical initiation[8] and have also shown that the rate of nucleotide reduction can be studied in an informative fashion by site-specific replacement of Y356 with a difluorotyrosine.[9] We now present the first studies of site-specific replacement of Y356 with a nontyrosine-based amino acid probe, which can be used to distinguish the ordering of proton versus electron transfer.

The aniline amino acid, *p*-aminophenylalanine ($PheNH_2$) (Figure 2A), provides a powerful mechanistic probe for enzymatic reactions involving redox-active tyrosines[10] because the aniline side chain cannot be deprotonated in the biologically relevant regime[11] prior to oxidation, yet has a similar reduction potential at neutral pH.[12,13] The titration curve for *N*-acetyl-*p*-aminophenylalanine methyl ester

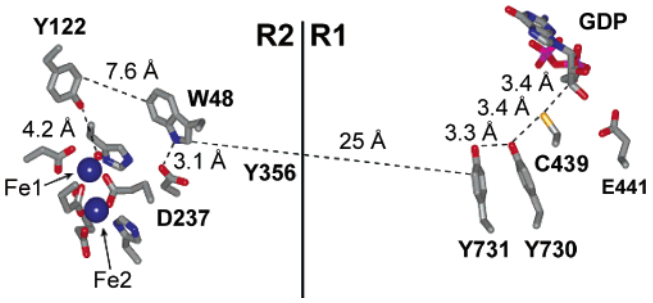

Figure 1. Radical initiation pathway of conserved aromatic amino acids.

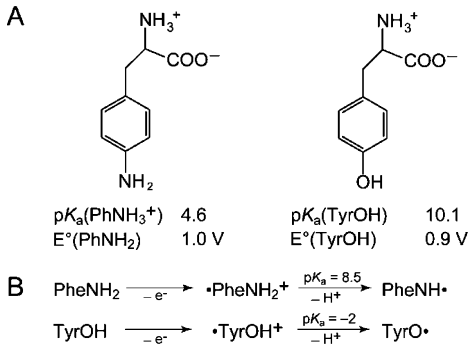

Figure 2. Comparison of $PheNH_2$ and tyrosine (refs 11−15). (A) pK_a and reduction potential. The data associated with $PheNH_2$ is given for aniline ($PhNH_2$). (B) Cation radical pK_a. Data for $PheNH_2$ is given for *p*-methylaniline.

(Ac-$PheNH_2$-OMe) indicates a protonation event with a pK_a of 4.6, which is nearly identical to aniline (see Supporting Information). In addition, the pK_a of the aniline cation radical is within the physiological range,[12] allowing equilibration of the proton at a physiological pH, unlike the corresponding tyrosyl radical (Figure 2B).[14] Thus, replacement of Y356 with $PheNH_2$ will allow us to address the sequence of proton versus electron transfer in the long-distance radical transfer pathway of the class I RNR from *E. coli* because nucleotide reduction will only be possible if ET occurs before proton transfer.

The pH dependence of the reduction potentials of the blocked amino acids, Ac-$PheNH_2$-OMe and *N*-acetyltyrosinamide (Ac-Tyr-NH_2), were measured by differential pulse voltammetry[15] (Figure 3). The potential difference between the $PheNH_2$ and Tyr in the physiological regime ranges from 60 mV at pH 6 to 160 mV at pH 9. This is within the range that we expect to be mechanistically informative, as previous studies have shown that redox potential differences of >200 mV can block radical transfer, and consequently nucleotide reduction, entirely.[8b] R2 was semisynthesized using intein technology[8b,16] with Y356 site-specifically replaced with $PheNH_2$ (see Supporting Information). The pH rate profile[8b] of Y356$PheNH_2$−R2 versus semisynthetic R2 was then generated

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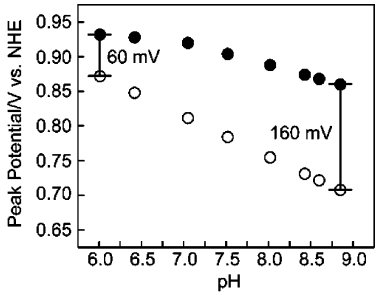

Figure 3. Peak potential of Ac-PheNH$_2$-OMe (●) and Ac-Tyr-NH$_2$ (○) as a function of pH measured by differential pulse voltammetry

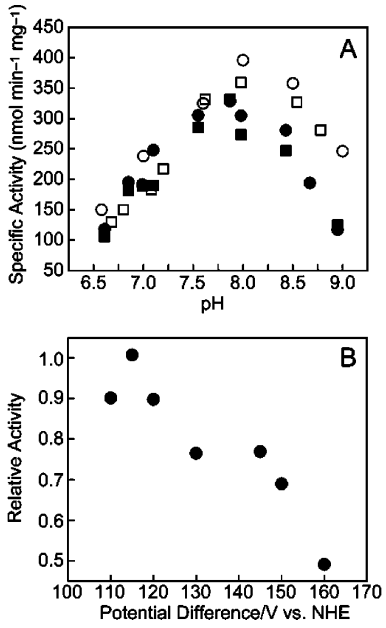

Figure 4. (A) pH rate profile of R2 semisynthesized with either PheNH$_2$ (set no. 1, ●; set no. 2, ■) or Tyr (set no. 1, ○; set no. 2, □) at position 356. (B) Plot of potential difference between Ac-PheNH$_2$-OMe and Ac-Tyr-NH$_2$ against the relative activity of Y356PheNH$_2$−R2 compared to semisynthetic R2.

(Figure 4A). The profile of Y356PheNH$_2$−R2 indicates that nucleotide reduction does indeed occur at rates similar to semisynthetic R2, with a drop-off in specific activity beginning at pH ~7.75. Thus, transfer of a proton prior to ET is not required for radical transfer through position 356. The activity drop also does not appear to be correlated with the pK_a's of either the Tyr or PheNH$_2$ cation radicals (Figure 2B). More specifically, the oxidized Tyr would be deprotonated, while PheNH$_2^{\bullet+}$ would remain protonated from pH 6.5 to 7.5 even though the specific activities are identical in that pH range. Additionally, loss of a proton from PheNH$_2^{\bullet+}$ occurs much less rapidly (millisecond time scale)[17] than from the corresponding Tyr cation radical, implying that radical transfer is not rate-limited by slowing proton release from the cation radical. Although it is possible that the pK_a may be perturbed, our studies with nitrotyrosine356−R2 suggest that the pK_a of residue 356 is not greatly changed in the R1−R2 complex compared to the pK_a of the amino acid in solution.[8b] Interestingly, the different hydrogen-bonding geometry does not seem to affect enzymatic activity, although the phenol side chain of tyrosine is a strong one-point hydrogen bond donor ($\alpha_2^H = 0.60$) while the aniline side chain of PheNH$_2$ provides a weak two-point hydrogen bond ($\alpha_2^H = 0.26$).[18] These results strongly support our previous conclusions from studies of 2,3-difluorotyrosine356−R2, which indicate that a proton may be lost from the pathway without altering nucleotide reduction activity.[9] Indeed, the simplest explanation for the pH rate profile of Y356PheNH$_2$−R2 is that the decrease in specific activity with increasing pH can be attributed to the increasing redox potential difference between PheNH$_2$ and Tyr and not by affecting the protonation state of residue 356 (Figure 4B), whether before or after the ET event. Thus, we infer that at a potential difference of >100 mV, the rate-limiting step in nucleotide reduction switches from a conformational change[19] to ET and that the redox activity of residue 356 can be directly interrogated (Figure 4B).[20]

PheNH$_2$ provides an effective mechanistic probe for redox-active tyrosines in biology. The replacement of a conserved tyrosine in the radical transfer pathway of the class I RNR with an aniline amino acid has allowed us to chemically resolve electron and proton transfer in a complex enzymatic system. We also propose that the rate of radical transfer through position 356 is affected mainly by the reduction potential of the residue at that position and not controlled by hydrogen bonding or proton transfer.

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Supporting Information Available: pK_a titration and DPVs of Ac-PheNH$_2$-OMe. RP-HPLC, MALDI-TOF MS, and UV spectrum of the 22-mer peptide. FPLC traces, SDS-PAGE gels, and ESI-MS from the semisynthesis of Y356PheNH$_2$−R2. This material is available free of charge via the Internet at http://pubs.acs.org.

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