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Use of 3 aminotyrosine to examine pathway dependence of radical propagation in *Escherichia coli* ribonucleotide reductase[†]

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Abstract

Escherichia coli ribonucleotide reductase (RNR), an $\alpha\beta_2$ complex, catalyzes the conversion of nucleoside 5'-diphosphate substrates (S) to 2'-deoxynucleoside 5'-diphosphates. α_2 houses the active site for nucleotide reduction and the binding site for allosteric effectors (E). β_2 contains the essential diferric tyrosyl radical ($Y_{122}^\bullet$) cofactor which, in the presence of S and E, oxidizes C_{439} in α to a thiyl radical, $C_{439}^\bullet$, to initiate nucleotide reduction. This oxidation occurs over 35 Å and is proposed to involve a specific pathway: $Y_{122}^\bullet \rightarrow W_{48} \rightarrow Y_{356}$ in β_2 to $Y_{731} \rightarrow Y_{730} \rightarrow C_{439}$ in α_2 . 3-Aminotyrosine (NH_2Y) has been site specifically incorporated at residues 730 and 731, and formation of the aminotyrosyl radical ($NH_2Y^\bullet$) has been examined by stopped-flow (SF) UV-vis and EPR spectroscopies. To examine the pathway dependence of radical propagation, the double-mutant complexes $Y_{356}F-\beta_2:Y_{731}NH_2Y-\alpha_2$, $Y_{356}F-\beta_2:Y_{730}NH_2Y-\alpha_2$, and $wt-\beta_2:Y_{731}F/Y_{730}NH_2Y-\alpha_2$, in which the non-oxidizable F acts as a pathway block, were studied by SF and EPR spectroscopies. In all cases, no $NH_2Y^\bullet$ was detected. To study off-pathway oxidation, Y_{413} , located 5 Å from Y_{730} and Y_{731} but not implicated in long-range oxidation, was examined. Evidence for $NH_2Y_{413}^\bullet$ was sought in three complexes: $wt-\beta_2:Y_{413}NH_2Y-\alpha_2$ (a), $wt-\beta_2:Y_{731}F/Y_{413}NH_2Y-\alpha_2$ (b), and $Y_{356}F-\beta_2:Y_{413}NH_2Y-\alpha_2$ (c). With (a), $NH_2Y^\bullet$ was formed with a rate constant of 25–30% and an amplitude of 25% that observed for its formation at residues 731 and 730. With (b), the rate constant for $NH_2Y^\bullet$ formation was 0.2–0.3% that observed at 731 and 730, and with (c), no $NH_2Y^\bullet$ was observed. These studies suggest the evolution of an optimized pathway of conserved Ys in the oxidation of C_{439} .

Long-range electron transfer (ET) (1) is prevalent in biology and plays a central role in many processes including respiration (2), nitrogen fixation (3), and the photosynthetic reaction centers (4). Studies over many decades have established that ET occurs rapidly and efficiently between metal clusters that are spaced 10 to 15 Å apart (5) and that the process involves electron tunneling as described by the Marcus-Levich equation (6). The importance of the medium, the intervening polypeptide structural motifs, has been studied extensively in

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SUPPORTING INFORMATION AVAILABLE Stopped-flow traces, EPR reaction spectra and corresponding subtractions, and SDS-PAGE of purified $Y_{413}NH_2Y-\alpha_2$ are included, as indicated in the article text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

model proteins and biological systems (1,7). The protein fold clearly plays a central role in lowering the reorganization energy of the biological ET reaction. It is also clear that ET kinetics can be regulated by the dynamics of conformational changes, especially across protein-protein interfaces (8,9).

The discovery of the central role of a tyrosyl radical ($Y\bullet$) in the class Ia ribonucleotide reductase (10) and the O_2 -evolving complex of photosystem II (11) led to the realization that ET processes over long distances are not limited to metal clusters. Long-range ET can also involve the aromatic amino acids tryptophan and tyrosine, the oxidations of which require loss of both an electron and a proton (12). Ribonucleotide reductase (RNR), which catalyzes the conversion of nucleotides to deoxynucleotides (13), has served as the paradigm for long-range proton-coupled electron transfer (PCET) with the tyrosyl radical ($Y\bullet$) in the β subunit mediating the oxidation of a cysteine in the α subunit 35 Å removed (Figure 1) (12,14). The present paper describes the use of 3-aminotyrosine (NH_2Y), site specifically incorporated into the α subunit of RNR (15), to examine the pathway dependence, and the importance of transient intermediates, proposed for this oxidation.

Tyrosyl radicals ($Y\bullet$) and tryptophan radicals ($W\bullet$) have been studied in three different contexts indirectly related to radical propagation in RNRs. In the area of model proteins and peptides, oxidizable amino acids have been positioned between electron donor/acceptor pairs. Studies of the ET process in the resulting systems have demonstrated rate accelerations associated with a hopping model requiring formation of a transient intermediate (16,17). These “simple” systems have allowed detailed mechanistic analyses and provide precedent for the multiple hopping steps proposed for RNR.

Y and W radicals have been observed in heme and non-heme iron dependent proteins, in which a reactive metal-based intermediate generated during catalysis is, in the absence of substrate, reduced by ET from an aromatic residue(s) in the vicinity of the metallocofactor (18-21). In general, low amounts of multiple radical species are generated on a slow (second) time scale. These systems are starting to define the mechanisms and consequences of aberrant oxidations and provide a model for the phenotype of an off-pathway oxidation in RNR.

Finally, transient aromatic amino acid radicals have been studied in enzymes such as cytochrome *c* peroxidase and prostaglandin synthase, in which metallocofactors are responsible for the oxidation of aromatic amino acids over short distances in the presence of substrate (22,23). Only ribonucleotide reductase and photolyase (PL) however are believed to utilize multiple, transient aromatic amino acid radical intermediates in long-range ET. PL, as isolated, contains a flavin cofactor in the semiquinone form ($FADH\bullet$) that needs to be reduced to its active form ($FADH^-$) prior to catalysis (24). Time-resolved kinetic studies suggest that the reduction occurs by a hopping mechanism involving three conserved Ws over a 15 Å distance (25). To obtain insight into the pathway dependence of reduction of $FADH\bullet$, the non oxidizable amino acid phenylalanine (F) was incorporated in place of each of the Ws and re-analyzed by ultrafast kinetics (26-28). The measurements on the wild-type and mutant PLs support a model in which 3 Ws act as a wire to the flavin cofactor.

The class Ia ribonucleotide reductases are the only known enzymes whose physiological function requires long-range oxidation, thought to occur in a pathway-dependent fashion through a series of aromatic amino acids (Figure 1). This provocative mechanism was first proposed by Uhlin and Eklund on the basis of a docking model of the $\alpha 2:\beta 2$ complex and on the strict conservation of the residues in primary sequence alignments (14). Mutagenesis studies in which each of the residues was replaced individually by F demonstrated their importance in catalysis, structure, or both (29-32). However, only with the advent of

technology for the site-specific incorporation of unnatural amino acids into α and β has the involvement of the three tyrosines (356 in $\beta 2$, 731 and 730 in $\alpha 2$, Figure 1) and the distance of the individual oxidation “steps” been examined (15,33-36). We have demonstrated recently the site-specific incorporation of 3-aminotyrosine (NH_2Y) into the α and β subunits at each of these positions. SF UV-vis and EPR studies revealed that $\text{NH}_2\text{Y}^\bullet$ is generated in a nucleotide-dependent manner with multiphasic kinetics, and that the rates of formation are maximized when substrate and effectors are bound (15,37). In addition, our studies indicated that NH_2Y - α s are capable of supporting nucleotide reduction, suggesting that the observed radical may be an intermediate on the proposed reaction pathway. From these collective studies, we have proposed that radical propagation occurs via orthogonal PCET in the $\beta 2$ subunit, with the proton transferring off-pathway to an amino acid acceptor or to solvent, and via co-linear PCET in the $\alpha 2$ subunit, with both proton and electron transferring between the same donor/acceptor pair (12,15).

Despite the appeal of a conserved PCET pathway, evidence in support of transient radical intermediates in RNR has been challenging to obtain because the reaction is rate-limited by conformational changes gated by binding of substrates and effectors to α (38). In previous efforts to induce light-mediated turnover on a photopeptide: $\alpha 2$ RNR complex, we have observed deoxynucleotide formation and transient formation of a $\text{Y}^\bullet$ within the peptide, but due to conformational gating, we have thus far been unable to detect transient intermediates within α (39,40).

NH_2Y is easier to oxidize than Y by 190 mV at pH 7 (15). From a thermodynamic perspective, the detection of $\text{NH}_2\text{Y}^\bullet$ in NH_2Y - α s may not be entirely surprising. Thus the pathway dependence of $\text{NH}_2\text{Y}^\bullet$ formation is important to establish (Figure 1). To provide further support for a defined pathway, F has been incorporated in place of each Y in the pathway to function as a block of $\text{NH}_2\text{Y}^\bullet$ formation. Incubation of Y_{356}F - $\beta 2$: $\text{NH}_2\text{Y}_{731}$ - $\alpha 2$, Y_{356}F - $\beta 2$: $\text{NH}_2\text{Y}_{730}$ - $\alpha 2$, or wt- $\beta 2$: Y_{731}F / $\text{NH}_2\text{Y}_{730}$ - $\alpha 2$ with CDP/ATP completely blocks NH_2Y oxidation, supporting the importance of a specific pathway. As a further test of the pathway dependence, the residue Y_{413} was selected for site specific “off-pathway” NH_2Y incorporation due to its proximity to Y_{731} and Y_{730} in α . Analogous studies were conducted with wt- $\beta 2$: $\text{Y}_{413}\text{NH}_2\text{Y}$ - $\alpha 2$, wt- $\beta 2$: $\text{Y}_{731}\text{F}/\text{Y}_{413}\text{NH}_2\text{Y}$ - $\alpha 2$, and Y_{356}F - $\beta 2$: $\text{Y}_{413}\text{NH}_2\text{Y}$ - $\alpha 2$ in which evidence for $\text{NH}_2\text{Y}^\bullet$ formation was sought by SF and EPR spectroscopy. The results of these experiments establish that functional long-range PCET in RNR occurs via an optimized pathway of amino acids spanning >35 Å across the α : β interface.

MATERIALS AND METHODS

Materials

The expression and purification of wt- $\alpha 2$ (2500 nmol/min/mg), wt- $\beta 2$ (1.2 $\text{Y}_{122}^\bullet$ /dimer, 7600 nmol/min/mg), $\text{Y}_{731}\text{NH}_2\text{Y}$ - $\alpha 2$ (150 nmol/min/mg), $\text{Y}_{730}\text{NH}_2\text{Y}$ - $\alpha 2$ (110 nmol/min/mg), His- Y_{356}F - $\beta 2$ (1.0-1.2 $\text{Y}_{122}^\bullet$ /dimer, <1 nmol/min/mg), and Y_{730}F - $\alpha 2$ (<25 nmol/min/mg) were conducted as previously described (15,41). His- Y_{356}F - $\beta 2$ will be referred to as “ Y_{356}F - $\beta 2$ ” henceforth. *E. coli* thioredoxin (TR, 40 units/mg) and thioredoxin reductase (TRR, 1400 units/mg) were isolated as previously described (41). [$5\text{-}^3\text{H}$]-CDP was purchased from ViTrax (Placentia, CA).

Generation of pTrc-nrdA-TAG₇₃₀TTT₇₃₁, pTrc-nrdA-TAG₄₁₃ and pTrc-nrdA-TAG₄₁₃TTT₇₃₁

The QuikChange Kit (Stratagene) was used according to manufacturer’s instructions to generate pTrc *nrdA*-TAG₇₃₀TTT₇₃₁, pTrc-*nrdA*-TAG₄₁₃ and pTrc-*nrdA*-TAG₄₁₃TTT₇₃₁. To generate pTrc-*nrdA*-TAG₇₃₀TTT₇₃₁, the template, pTrc-*nrdA*-TAG₇₃₀, (15) was amplified in the presence of forward (5'-TTC GGG GTC AAA ACA CTG TAG TTT CAG AAC ACC

CGT GAC GGC GCT-3') and reverse (5'-AGC GCC GTC ACG GGT GTT CTG AAA CTA CAG TGT TTT GAC CCC GAA-3') primers to insert a TTT codon (Phe) at residue 731. To generate pTrc-*nrdA*-TAG₄₁₃, the template, pTrc-*nrdA*-wt, was amplified in the presence of forward (5'-GCG TCT ACC GGT CGT ATC TAG ATT CAG AAC GTT GAC CAC TGC-3') and reverse (5'-GCA GTG GTC AAC GTT CTG AAT CTA GAT ACG ACC GGT AGA CGC-3') primers to insert the amber stop codon (TAG) at residue 413. pTrc-*nrdA*-TAG₄₁₃TTT₇₃₁ was then generated from pTrc-*nrdA*-TAG₄₁₃, with forward (5'-C GGG GTC AAA ACA CTG TAT TTT CAG AAC ACC CGT GAC G-3') and reverse (5'-C GTC ACG GGT GTT CTG AAA ATA CAG TGT TTT GAC CCC G-3') primers to insert a TTT codon (Phe) at residue 731. For each plasmid, the mutation(s) was confirmed by sequencing the entire gene at the MIT Biopolymers Laboratory.

Expression, purification, and activity determination of Y₇₃₁F/Y₇₃₀NH₂Y- α 2

Y₇₃₁F/Y₇₃₀NH₂Y- α 2 was expressed, purified, and pre-reduced as described for Y₇₃₀NH₂Y- α 2 and Y₇₃₁NH₂Y- α 2 (15). Specific activity was determined by the radioactive RNR assay (33). Assays were conducted with mutant α 2 and wt β 2 at concentrations of 0.5 μ M and 2.5 μ M, respectively, in 50 mM HEPES, 15 mM MgSO₄, 1 mM EDTA, pH 7.6 (assay buffer) at 25 °C. [5-³H]-CDP had a specific activity of 1450 cpm/nmol. The reaction product was dephosphorylated by treatment with alkaline phosphatase and the resulting dC was quantitated by the method of Steeper and Stuart (33).

Expression, purification, and activity determination of Y₄₁₃NH₂Y- α 2 and Y₇₃₁F/Y₄₁₃NH₂Y- α 2

Y₄₁₃NH₂Y- α 2 and Y₇₃₁F/Y₄₁₃NH₂Y- α 2 were expressed and purified as described for Y₇₃₀NH₂Y- α 2 and Y₇₃₁NH₂Y- α 2 (15). However, an additional step using a Poros HQ/20 FPLC anion exchange column (Applied Biosystems, 1.6 $\times$ 10 cm, 20 mL) was required to obtain protein of >95% purity judged by SDS-PAGE analysis. The column was equilibrated in 50 mM Tris, 1 mM EDTA, and 5 mM DTT, pH 7.6 and was loaded with 15-20 mg of Y₄₁₃NH₂Y- β 2 or Y₇₃₁F/Y₇₃₀NH₂Y- α 2. The column was washed with one column volume of equilibration buffer at a flow rate of 4 mL/min, then eluted with a linear gradient of 100 to 500 mM NaCl (60 mL $\times$ 60 mL) in the same buffer at the same flow rate. In the case of Y₇₃₁F/Y₇₃₀NH₂Y- α 2, the protein was chromatographed twice. Subsequent to the FPLC purification step, the protein was pre-reduced according to the standard procedure (15).

The activities of Y₄₁₃NH₂Y- α 2 and Y₇₃₁F/Y₄₁₃NH₂Y- α 2 were determined using the spectrophotometric (41) and/or radioactive assays (33), with minor modifications. Mutant α 2s (0.1 or 0.2 μ M) were assayed in the presence of a 5-fold excess of β 2 (0.5 μ M or 1.0 μ M) in assay buffer at 25 °C. [5-³H]-CDP had activity between 2700 and 4800 cpm/nmol.

Determination of the K_d for Y₄₁₃NH₂Y- α 2 and β 2 interaction

The K_d between Y₄₁₃NH₂Y- α 2 and wt β 2 was determined using Y₇₃₀F- α 2 as a competitive inhibitor of nucleotide reduction (29,42). Y₄₁₃NH₂Y- α 2 (0.7 μ M) and β 2 (0.35 μ M) were incubated with CDP (1 mM), ATP (1.6 mM), TR (50 μ M), TRR (1 μ M), and NADPH (0.2 mM) in assay buffer. Inhibition of RNR activity (as measured by decrease in NADPH consumption) was determined in the presence of increasing Y₇₃₀F- α 2 (0.1 μ M to 3 μ M). The data analysis for this procedure typically relies on the mutant α 2 of unknown K_d acting as the inhibitor of nucleotide reduction (36). The analysis was modified, as the K_d of the active Y₄₁₃NH₂Y- α 2 is not known, whereas the K_d of the inhibitor (Y₇₃₀F- α 2) had been previously determined under conditions in which the K_i and K_d of the inhibitor are equivalent (30). The K_d for the Y₄₁₃NH₂Y- α 2:wt- β 2 complex was approximated and the experimentally determined relative activities were then used to extrapolate the K_d for the Y₇₃₀F- α 2:wt- β 2 complex. The K_d for Y₄₁₃NH₂Y- α 2 was refined by iterative fitting of the experimental data until the fit yielded a K_d for Y₇₃₀F- α 2 that was in good agreement with the literature value.

Reaction of $\text{NH}_2\text{Y-}\alpha 2\text{s}$ with $\text{Y}_{356}\text{F-}\beta 2$ monitored by EPR spectroscopy

Pre reduced $\text{Y}_{730}\text{NH}_2\text{Y-}\alpha 2$ or $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$ (15 μM , final concentration) was mixed with $\text{Y}_{356}\text{F-}\beta 2$ (15 μM , 1 $\text{Y}\cdot/\beta 2$), and CDP/ATP (1 mM/3 mM, respectively) in assay buffer at 25 °C. The reactions were quenched at 20 s or 2 min by hand in liquid N_2 . The reaction of $\text{Y}_{413}\text{NH}_2\text{Y-}\alpha 2$ and $\text{Y}_{356}\text{F-}\beta 2$ was conducted similarly, except the final protein concentration was 50 μM . EPR spectra were recorded at 77 K on a Bruker ESP-300 X-band spectrometer equipped with a quartz finger dewar containing liquid N_2 in the Department of Chemistry Instrumentation Facility. EPR parameters were as follows: microwave frequency=9.34 GHz, power=30 μW , modulation amplitude=1.5 G, modulation frequency=100 kHz, time constant=5.12 ms, scan time=41.9 s. EPR spin quantitation was carried out in WinEPR (Bruker) using Cu^{II} as a standard (43). EPR subtractions were conducted with an in-house, Excel-based program using the spectrum of $\text{Y}_{122}\cdot$ from either wt $\beta 2$ or a wt- $\beta 2$:mutant- $\alpha 2$ complex as a reference.

Reaction of $\text{Y}_{356}\text{F-}\beta 2$ with $\text{NH}_2\text{Y-}\alpha 2\text{s}$ monitored by SF UV-vis spectroscopy

SF kinetics were performed on an Applied Photophysics DX 17MV instrument equipped with the Pro-Data upgrade. Pre-reduced $\text{Y}_{730}\text{NH}_2\text{Y-}\alpha 2$ (or $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$) and ATP were mixed with $\text{Y}_{356}\text{F-}\beta 2$ and CDP to yield final concentrations of 8 μM , 3 mM, 8 μM and 1 mM, respectively, in assay buffer at 25 °C. The concentrations of $\text{Y}_{122}\cdot$, $\text{NH}_2\text{Y}_{731}\cdot$ and $\text{NH}_2\text{Y}_{730}\cdot$ were monitored at 410 nm ($\epsilon = 3,700 \text{ M}^{-1} \text{ cm}^{-1}$), 320 nm ($\epsilon = 11,000 \text{ M}^{-1} \text{ cm}^{-1}$) and 325 nm ($\epsilon = 10,500 \text{ M}^{-1} \text{ cm}^{-1}$), respectively, using PMT detection. In each experiment, 5–7 traces were averaged and kinetic parameters obtained by curve fitting using OriginPro or Kaleidagraph software. Iterative rounds of fitting were carried out until both the residual plot and the r^2 correlation value were optimized.

Reaction of $\text{Y}_{731}\text{F/Y}_{730}\text{NH}_2\text{Y-}\alpha 2$, $\text{Y}_{413}\text{NH}_2\text{Y-}\alpha 2$, and $\text{Y}_{731}\text{F/Y}_{413}\text{NH}_2\text{Y-}\alpha 2$ with wt $\beta 2$ monitored by EPR and SF UV-vis spectroscopies

$\text{Y}_{731}\text{F/Y}_{730}\text{NH}_2\text{Y-}\alpha 2$, $\text{Y}_{413}\text{NH}_2\text{Y-}\alpha 2$, or $\text{Y}_{731}\text{F/Y}_{413}\text{NH}_2\text{Y-}\alpha 2$ was reacted with wt $\beta 2$ in the presence of CDP/ATP at 25 °C as described for $\text{NH}_2\text{Y-}\alpha 2\text{s}$ above. For the EPR and SF experiments, $\alpha 2$ and $\beta 2$ concentrations were 50 μM and 5 μM , respectively. The EPR reactions were hand quenched at 40 s. The EPR spectrum of a putative $\text{NH}_2\text{Y}_{413}\cdot$ was obtained subsequent to subtraction of the spectrum of $\text{Y}_{122}\cdot$ (in the wt- $\beta 2$:mutant- $\alpha 2$ complex) as described above.

RESULTS

Reaction of $\text{Y}_{730} \text{NH}_2\text{Y-}\alpha 2$ (or $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$) with $\text{Y}_{356}\text{F-}\beta 2$ monitored by EPR and SF spectroscopies

It was previously demonstrated that reaction of either $\text{Y}_{730}\text{NH}_2\text{Y-}\alpha 2$ or $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$ with wt- $\beta 2$, CDP, and ATP resulted in the loss of $\text{Y}_{122}\cdot$ ($\lambda_{\text{max}} = 410 \text{ nm}$, g_{av} of 2.0047) concomitant with formation of a new radical species, assigned as $\text{NH}_2\text{Y}\cdot$ on the basis of its absorbance and EPR spectroscopic features ($\lambda_{\text{max}} = 320\text{--}325 \text{ nm}$, g_{av} of 2.0043) (15). In this paper, experiments with $\text{Y}_{356}\text{F-}\beta 2$, in which the radical propagation pathway is disrupted with a Y to F mutation, were conducted to establish that trapping of $\text{NH}_2\text{Y}\cdot$ at residues 730 and 731 is the result of the participation of these residues in radical transfer, rather than the consequence of introducing a pathway-independent thermodynamic trap into $\alpha 2$. If formation of $\text{NH}_2\text{Y}_{730}\cdot$ or $\text{NH}_2\text{Y}_{731}\cdot$ requires a redox-active Y_{356} residue, then no $\text{NH}_2\text{Y}\cdot$ should be observed. Reactions were carried out with CDP/ATP, $\text{Y}_{356}\text{F-}\beta 2$ and $\text{Y}_{730} \text{NH}_2\text{Y-}\alpha 2$ (or $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$) and quenched at 77 K after 20 s and 2 min. Analysis of the EPR spectra, subsequent to subtraction of the spectrum of the resting $\text{Y}_{122}\cdot$ at time zero gave no evidence of $\text{NH}_2\text{Y}\cdot$ at either time point for reactions with $\text{Y}_{730}\text{NH}_2\text{Y-}\alpha 2$ or with

$Y_{731}NH_2Y-\alpha 2$ (Figure S1). In addition, spin quantitation of the signal at time zero in comparison to times 20 s and 2 min revealed no significant loss of spin. A control with non tagged $Y_{356}F-\beta 2$ gave the same result as that observed with His- $Y_{356}F-\beta 2$, demonstrating that the His-tag does not interfere with radical formation. Oxidation of Y_{356} is thus a prerequisite for hole migration into the $\alpha 2$ subunit.

To determine if $NH_2Y\bullet$ was formed transiently, the reactions of $NH_2Y-\alpha 2$ s with $Y_{356}F-\beta 2$, CDP, and ATP were monitored by SF UV-vis spectroscopy at 325 nm (λ_{max} of $NH_2Y_{730}\bullet$) or 320 nm (λ_{max} of $NH_2Y_{731}\bullet$) and at 410 nm (λ_{max} of $Y_{122}\bullet$). The results for $Y_{730}NH_2Y-\alpha 2$ with $Y_{356}F-\beta 2$ are shown in Figure 2, and for $Y_{731}NH_2Y-\alpha 2$ with $Y_{356}F-\beta 2$ in Figure S2. A small increase at 325 (320 nm) corresponding to $\sim 2\%$ of total initial $Y_{122}\bullet$ was observed. However, these spectral changes were not correlated with the expected decrease in absorbance at 410 nm due to loss of the $Y\bullet$. In fact, a small increase in this wavelength was observed (Figures 2 & Figure S2, *inset*). The lack of correspondence between the spectral changes at 325 (or 320) nm and 410 nm indicates these features are not related to $NH_2Y\bullet$ formation. The nature of these changes is not understood at present, but similar changes have been observed previously under different reaction conditions (37) and may be related to minor structural changes of the $Y_{122}\bullet$ diferric cluster upon $\alpha 2:\beta 2$ complex formation (Seyedsayamdost, Minnihan, and Stubbe, unpublished results). The SF UV-vis and EPR spectroscopic data together indicate that no $NH_2Y\bullet$ is formed at residues 730 or 731 with $Y_{356}F-\beta 2$, with our lower limit of detection approximated as less than 2% (by SF UV-vis) and less than 3% (by EPR) of the total initial $Y_{122}\bullet$. These data support the conclusion that a redox-active residue at 356 is essential for radical transfer into $\alpha 2$.

Reaction of $Y_{731}F/Y_{730}NH_2Y-\alpha 2$ with wt- $\beta 2$ monitored by EPR and SF spectroscopies

To assess the role of residue Y_{731} in the radical transfer chain, the double mutant $Y_{731}F/Y_{730}NH_2Y-\alpha 2$ was isolated, characterized, and examined for $NH_2Y\bullet$ formation. The protein was purified to homogeneity and its specific activity measured to be 36 nmol/min/mg. This low activity, approximately 1.4% that of the wild-type enzyme, is likely associated with co-purifying endogenous $\alpha 2$, rather than activity inherent to the mutant protein. This result is in agreement with the activity of $Y_{731}F-\alpha 2$ (26 nmol/min/mg) and contrasts to activities measured in $Y_{730}NH_2Y-\alpha 2$ or $Y_{731}NH_2Y-\alpha 2$ (110-150 nmol/min/mg) (15).

$Y_{731}F/Y_{730}NH_2Y-\alpha 2$ and ATP were mixed with wt- $\beta 2$ and CDP and the reaction examined by EPR and SF spectroscopy. With EPR, the reactions were quenched at 40 s or 2 min and analyzed as described above. No $NH_2Y\bullet$ formation was detected in either case and the total spin relative to starting $Y_{122}\bullet$ remained unchanged (Figure S3). The SF UV-vis experiments gave results similar to those observed with $Y_{356}F-\beta 2$ at both 325 nm and 410 nm (Figure S3). Thus formation of $NH_2Y_{730}\bullet$ also requires a redox active Y_{731} for radical propagation.

Expression, purification, and characterization of $Y_{413}NH_2Y-\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y-\alpha 2$

As an additional test of the pathway dependence of $NH_2Y\bullet$ formation at Y_{730} and Y_{731} , NH_2Y was site-specifically incorporated at a Y in $\alpha 2$ that is thought not to participate in the radical transfer pathway (Figure 1). Residue Y_{413} was selected, as the X-ray structure of *E. coli* $\alpha 2$ (14) reveals distances of 5.0 Å or 5.2 Å between the phenolic oxygen of Y_{413} and that of Y_{730} or Y_{731} , respectively (Figure 3). Thus, Y_{413} is a distance from Y_{730} and Y_{731} that is reasonable for ET between these residues. Y_{731} and Y_{730} are separated by 3.3 Å in the same structure. Alignment of 142 primary sequences of class Ia/b $\alpha 2$ s reveals that while 413 is not conserved, it is always an aromatic amino acid. Y, F, and W occupy this position 65%, 21%, and 14% of the time, respectively. Thus, the proteins $Y_{413}NH_2Y-\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ were expressed and purified to be examined for $NH_2Y\bullet$ formation.

$Y_{413}NH_2Y-\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ were expressed in a fashion analogous to other NH_2Y -substituted $\alpha 2$ s. Their purification, however, proved more difficult than for previous NH_2Y -substituted $\alpha 2$ s. Protein of high purity (as judged by SDS-PAGE) was eventually isolated via anion-exchange FPLC with an optimized salt gradient, but at the expense of overall protein yield (Figure S4).

The activities of the purified mutants were determined by the spectrophotometric and/or radioactive RNR assays. The specific activity of $Y_{413}NH_2Y-\alpha 2$ was 46% of wt $\alpha 2$ (~1200 nmol/min/mg), while that of $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ was 1.9% of wt $\alpha 2$ (~48 nmol/min/mg). The activity of $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ is similar to that of $Y_{731}F/Y_{730}NH_2Y-\alpha 2$, and is likely associated with endogenous *E. coli* $\alpha 2$ that co-purified with the recombinantly expressed mutant protein.

The reduction in activity of $Y_{413}NH_2Y-\alpha 2$ relative to wt $\alpha 2$, the proximity of Y_{413} to the putative $\alpha 2:\beta 2$ interface, and the difficulty encountered in purification of the related mutants provided the impetus to determine the K_d for the interaction between this mutant and $\beta 2$ using a modified version of the competitive inhibition assay developed by Climent et al. (29,36,42). Data analysis was modified relative to the previously published method (36) to accommodate for the condition that the active species, rather than the inhibitor, was also the species of unknown K_d (Figure S5). Analysis by this method afforded a K_d of 0.05 μM , similar to the value of 0.06 μM for wt- $\alpha 2:\beta 2$ in the presence of CDP and ATP (Hassan and Stubbe, unpublished results). This finding, in conjunction with the activity of $Y_{413}NH_2Y-\alpha 2$, suggests that $Y_{413}NH_2Y-\alpha 2$ is folded and that binding between subunits is minimally perturbed. However, attempts to crystallize $Y_{413}NH_2Y-\alpha 2$ under conditions optimized for wt- $\alpha 2$ and successfully used to crystallize other $NH_2Y-\alpha 2$ mutants failed to provide crystals of $Y_{413}NH_2Y-\alpha 2$, suggesting that mutation of Y_{413} produces some effect on protein stability or solubility (Uhlin and Stubbe, unpublished results).

Reactions of $Y_{413}NH_2Y-\alpha 2$ or $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ with wt- $\beta 2$, and $Y_{413}NH_2Y-\alpha 2$ with $Y_{356}F-\beta 2$ monitored by EPR spectroscopy

EPR experiments were carried out as described above by mixing $Y_{413}NH_2Y-\alpha 2$ (or $Y_{731}F/Y_{413}NH_2Y-\alpha 2$) with CDP, ATP and wt- $\beta 2$, or $Y_{413}NH_2Y-\alpha 2$ with CDP, ATP, and $Y_{356}F-\beta 2$. Each reaction was quenched at 40 s. The resulting spectra, subsequent to subtraction of the resting $Y_{122}\bullet$ in the mutant $\alpha 2:\beta 2$ complex, are shown in Figure 4, and the spin quantitation of the resulting $NH_2Y\bullet$ is given in Table 1. In the reactions of $Y_{413}NH_2Y-\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ with wt- $\beta 2$ (Figure 4a and 4b, respectively), the difference EPR spectrum reveals features characteristic of an $NH_2Y\bullet$. For the reaction with $Y_{413}NH_2Y-\alpha 2$, the signal attributed to $NH_2Y\bullet$ accounts for 12% of the total spin. Differences in the spectral features relative to those previously reported $NH_2Y_{730}\bullet$ and $NH_2Y_{731}\bullet$ cannot be assigned at this stage, but perhaps report on differences in the dihedral angles of the C_β -protons on NH_2Y at position 413 versus those at positions 730 and 731. For the reaction with $Y_{731}F/Y_{413}NH_2Y-\alpha 2$, the signal attributed to $NH_2Y\bullet$ accounts for 7% of the total spin. The magnitude of the signal in both cases is substantially lower than the “on pathway” radicals, which have been shown by EPR to constitute 53% and 45% of the total spin at the same time point when NH_2Y is incorporated at 730 or 731, respectively (15). The difference EPR spectrum from the reaction of $Y_{413}NH_2Y-\alpha 2$ with $Y_{356}F-\beta 2$ with CDP/ATP quenched at 40 s reveals no detectable $NH_2Y\bullet$ (Figure 4c).

Reactions of $Y_{413}NH_2Y-\alpha 2$ or $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ with wt- $\beta 2$ monitored by SF UV-vis spectroscopy

Given the results of the EPR experiments above, the kinetics of formation of the $NH_2Y_{413}\bullet$ were assessed for both $Y_{413}NH_2Y-\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ using SF UV-vis

spectroscopy. The results with $Y_{413}NH_2Y\text{-}\alpha 2$ and $Y_{731}F/Y_{413}NH_2Y\text{-}\alpha 2$ are shown in Figure 5a and 5b, respectively, and are summarized in Table 1. For the reaction with $Y_{413}NH_2Y\text{-}\alpha 2$ monitored at 325 nm, the observed absorbance increase may be fit to biphasic kinetics with rate constants of 26.4 s^{-1} and 4.2 s^{-1} . These phases correspond to 30% and 70%, respectively, of the total absorbance increase observed at 325 nm over the reaction course. The faster phase is not correlated, however, with a decrease at 410 nm, and thus is not associated with $NH_2Y\bullet$ formation.

The $A_{410\text{ nm}}$ decrease can be fit to biphasic kinetics with rate constants of 5.3 s^{-1} at 410 nm and 0.5 s^{-1} , corresponding to 47% and 53% of the total absorbance change at 410 nm during the reaction. The rate constant of 5.3 s^{-1} agrees well with the rate constant of 4.2 s^{-1} at 325 nm, and together suggest the formation of $NH_2Y_{413}\bullet$. Using the extinction coefficients of $NH_2Y\bullet$ and $Y\bullet$, the extent of formation of $NH_2Y_{413}\bullet$ was determined to be 8% of the starting $Y_{122}\bullet$. This is substantially lower than the amount of $NH_2Y\bullet$ observed in SF UV-vis studies with NH_2Y at 730 (39%) and 731 (35%) under analogous reaction conditions (15). While a rate constant of $4\text{--}5\text{ s}^{-1}$ is fast enough to be involved in production of dNDPs, the collective data on the three Y_{413} mutant complexes suggest $NH_2Y_{413}\bullet$ formation is off-pathway and we propose that it is not functionally relevant to catalysis.

For the reaction of $Y_{731}F/Y_{413}NH_2Y\text{-}\alpha 2$, a slow increase in $A_{325\text{ nm}}$ was observed concomitant with a loss of absorbance at 410 nm (Figure 5b). The first 30 s of data collected at both wavelengths were fit to a monoexponential function with a rate constant of 0.04 s^{-1} . The SF data suggest that 12% of the starting $Y_{122}\bullet$ is converted to $NH_2Y_{413}\bullet$ over the course of 30 s. The slow rate of radical formation, 320 to 475-fold slower than $NH_2Y\bullet$ formation at 730 (13 s^{-1}) or 731 (19 s^{-1}), suggest off-pathway oxidation.

DISCUSSION

The rate and extent of $NH_2Y\bullet$ formation in the presence of the β subunit and the S/E pair CDP/ATP (when NH_2Y has been site-specifically incorporated into α at residue 730, 731, or 413) may be used as indicators of the participation of a specific residue in a defined pathway for C_{439} oxidation in RNR (Figure 1). We have previously established that formation of $NH_2Y\bullet$ is biphasic, giving rise to rate constants and amplitudes (as a percent of initial $Y_{122}\bullet$) for $NH_2Y\bullet$ formation of 19 s^{-1} (24%) and 2.7 s^{-1} (11%) at 731, and 13 s^{-1} (20%) and 2.5 s^{-1} (19%) at 730 (15). We have analyzed the kinetics of $NH_2Y\bullet$ formation with all S/E pairs and argue that the slower rate constant is kinetically competent for deoxynucleotide formation (Seyedsayamdost, Minnihan, and Stubbe, unpublished results). An unexpected observation of our initial studies with the 730- and 731-substituted proteins was the ability of these $NH_2Y\text{-}\alpha 2$ s to catalyze deoxynucleotide formation, suggesting that $NH_2Y\bullet$ at either position may be an intermediate on the pathway or may replace $Y_{122}\bullet$ as a radical initiator.

In our current studies, replacement of Y with the non-oxidizable F in the pathway at positions 356 in β or in 731 in β , and incorporation of NH_2Y within the pathway after the block, resulted in no detectable $NH_2Y\bullet$ by EPR analysis at 20 s or 2 min (Table 1). Since the EPR signal is always a composite of $Y\bullet$ and $NH_2Y\bullet$, a subtraction of the $Y\bullet$ signal is required for spin quantitation of the two species. The broadness of the $Y\bullet$ signal relative to the $NH_2Y\bullet$ makes this subtraction straightforward, with the lower limit of detection of an $NH_2Y\bullet$ estimated to be 3% of the total spin. It should be noted that cleaner subtractions are achieved when $Y_{122}\bullet$ in an $\alpha:\beta$ complex is used as the reference, as the hyperfine interactions of $Y_{122}\bullet$ in β appear to be subtly affected by complex formation with α .

To establish that $NH_2Y\bullet$ is not generated transiently in the presence of a Y to F mutation, SF UV-vis was conducted and the reaction was monitored at 325 (320) nm and 410 nm. An

increase in the former absorption is associated with $\text{NH}_2\text{Y}\bullet$ formation at 730 (731) and a loss of the latter with $\text{Y}\bullet$ loss. Monitoring a reaction at 325 nm is complicated by features of the diferric cluster which also absorb in this region. Thus, we have assumed transient features observed by SF are related to $\text{NH}_2\text{Y}\bullet$ formation only if the rate constant and amplitude increase of the 325 nm feature parallels the rate constant and amplitude decrease of the 410 nm feature. As indicated in Figure 2, in all experiments described herein in which the pathway has been “blocked,” a small rise phase (approximately 2% of the $\text{Y}\bullet$) has been observed at 325 nm. This rise is rapid (corresponding to a rate constant $>20\text{ s}^{-1}$), has no corresponding decrease at 410 nm, and is attributed to a small change associated with the diferric $\text{Y}\bullet$ cofactor. In the current analysis, this feature is not considered further. Thus, the SF data are in agreement with the EPR results, indicating that a redox-active Y at 356 and 731 is a prerequisite for $\text{NH}_2\text{Y}\bullet$ formation.

As noted above, at pH 7.0 NH_2Y is easier to oxidize than Y by 190 mV and thus could potentially be oxidized in an off-pathway process. To test this proposal, studies were conducted in which NH_2Y was incorporated in place of a residue proximal to the proposed pathway. Y_{413} (Figure 3) is located within 5 Å of both Y_{731} and Y_{730} , a distance feasible for a single oxidation “hop,” and thus became the target of our off-pathway studies. Three experiments were carried out using mutants in which NH_2Y was site-specifically incorporated at position 413.

In the first, NH_2Y was incorporated at residue 413 while keeping all other on-pathway residues in tact. In two additional experiments, a pathway block was introduced in the form of a Y to F mutation at either 356 or 731. When the single mutant, $\text{Y}_{413}\text{NH}_2\text{Y}\text{-}\alpha 2$, was reacted with wt- $\beta 2$, CDP, and ATP, SF experiments revealed formation of $\text{NH}_2\text{Y}_{413}\bullet$ with a rate constant of $4\text{--}5\text{ s}^{-1}$, corresponding to an 8% conversion of the initial $\text{Y}_{122}\bullet$ to $\text{NH}_2\text{Y}_{413}\bullet$ (Table 1). EPR analysis of the same reaction after 40 s was in agreement with the SF results, indicating the formation of a $\text{NH}_2\text{Y}\bullet$ species accounting for 12% of the total spin (Figure 4a). The differences in the percent conversion of $\text{Y}_{122}\bullet$ to $\text{NH}_2\text{Y}_{413}\bullet$ as measured by SF and EPR may be indicative of the error inherent to the two methods in quantitation of low levels of radical species. Alternately, the differences may arise from the 10-fold difference in protein concentration used in the two experiments (38). As argued above, the rate constant and accumulation of $\text{NH}_2\text{Y}\bullet$ is indicative of the relevance of that specific residue to catalysis in RNR. The rate constant of 4.2 s^{-1} for $\text{NH}_2\text{Y}_{413}\bullet$ formation is slower by a factor of 4 and the amplitude is reduced by 75% relative to $\text{NH}_2\text{Y}\bullet$ formation at 731. This result suggests that NH_2Y at position 413 can be oxidized in an off-pathway fashion by either Y_{731} or by Y_{356} . With F incorporated at 731 and NH_2Y at 413, $\text{NH}_2\text{Y}\bullet$ is still observed. However, a fit of the SF data for the first 30 s gave a rate constant of 0.04 s^{-1} and an amplitude of 12%. EPR spin quantitation of the same reaction after 40 s indicated 7% of the total spin was associated with the putative $\text{NH}_2\text{Y}\bullet$ (Figure 4b). Finally, with F at 356 and NH_2Y at 413, no $\text{NH}_2\text{Y}\bullet$ was detected (Figure 4c). Thus the oxidation at 413 appears to occur predominantly through Y_{356} . The very slow rate constant for $\text{NH}_2\text{Y}_{413}\bullet$ formation with F at 731 ($\sim 0.2\%$ that of $\text{NH}_2\text{Y}\bullet$ formation at 731, and $\sim 2\%$ of the steady state-rate constant for dCDP formation) and the complete absence of $\text{NH}_2\text{Y}_{413}\bullet$ with F at 356 suggest that radical formation at 413 in the NH_2Y -mutants results from off-pathway ET.

It is experimentally challenging to determine directly whether $\text{NH}_2\text{Y}_{413}\bullet$ can participate as a chemically- and kinetically-competent intermediate in C_{439} oxidation. However, strong evidence that ET through 413 does *not* constitute a viable alternate radical pathway comes from the observation that no $\text{NH}_2\text{Y}\bullet$ is generated in the reaction with $\text{Y}_{731}\text{F}/\text{Y}_{730}\text{NH}_2\text{Y}\text{-}\alpha 2$. Y_{413} cannot serve as a bypass to a block at 731, and thus cannot support the oxidation of Y at 730 required for $\text{C}_{439}\bullet$ formation and subsequent nucleotide reduction. The $\text{NH}_2\text{Y}_{413}\bullet$

observed in experiments with Y₄₁₃NH₂Y- α 2 or Y₇₃₁F/Y₄₁₃NH₂Y- α 2 thus results from an off-pathway oxidation and is nonfunctional.

Many examples of off-pathway oxidation exist in the literature for heme- and non-heme iron-dependent systems. Likewise, evidence for off-pathway oxidation has been reported previously in the class I *E. coli* RNR (19-21,41). The phenotypes are usually slow rate constants for radical formation and low amplitudes of new radical species. Thus the experiments presented herein using F and NH₂Y as an oxidation block and trap, respectively, support the original proposal of Uhlin and Eklund for long-range oxidation of C₄₃₉ in α via a conserved pathway of defined aromatic amino acids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

α	ribonucleotide reductase large subunit
ATP	adenosine 5'-triphosphate
β	ribonucleotide reductase small subunit
C•	thiyl radical
CDP	cytidine 5'-diphosphate
dC	2'-deoxycytidine
DTT	dithiothreitol
E	effector
EPR	electron paramagnetic resonance
ET	electron transfer
FPLC	fast protein liquid chromatography
NH ₂ Y•	3-aminotyrosyl radical
PCET	proton-coupled electron transfer
PL	photolyase
RNR	ribonucleotide reductase
S	substrate
SF	stopped-flow
TR	thioredoxin
TRR	thioredoxin reductase
W•	tryptophan radical
wt	wild-type
Y•	tyrosyl radical

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FIGURE 1.

The proposed radical propagation pathway within an $\alpha:\beta$ pair of *E. coli* RNR. Y₃₅₆, Y₇₃₁, and Y₇₃₀ have been shown to be redox-active during radical transfer and their positions within the complex have been determined structurally (14) and/or experimentally (35). Y₃₅₆ is located in the structurally disordered C-terminal tail of β (44), and thus its location in the complex is not known.

FIGURE 2.

SF UV-vis spectroscopy of the reaction of $Y_{356}F\text{-}\beta 2$ with $Y_{730}NH_2Y\text{-}\alpha 2$ in the presence of CDP/ATP. The reaction was monitored at 325 nm (trace A, λ_{max} of $NH_2Y_{730}^*$) and at 410 nm (trace B, λ_{max} of Y_{122}^*). Insets show magnified views of the initial 0.5 s of each trace.

FIGURE 3.

Location of Y₄₁₃ (magenta) in relation to Y₇₃₁ (blue), Y₇₃₀ (blue), and C₄₃₉ (yellow) of the radical propagation pathway within the α subunit. Y₄₁₃ is located 5.0 and 5.2 Å from Y₇₃₀ and Y₇₃₁, respectively, with distances measured between phenolic oxygens (red). The figure was generated as a PyMol (www.pymol.org) rendition of PDB ID: 1RLR (14).

FIGURE 4.

Formation of $\text{NH}_2\text{Y}_{413}\bullet$ monitored by EPR spectroscopy. In each case, CDP and ATP were mixed at 25 °C with (A) wt $\beta 2$ and $\text{Y}_{413}\text{NH}_2\text{Y}-\alpha 2$, (B) wt $\beta 2$ and $\text{Y}_{731}\text{F}/\text{Y}_{413}\text{NH}_2\text{Y}-\alpha 2$, and (C) $\text{Y}_{356}\text{F}-\beta 2$ and $\text{Y}_{413}\text{NH}_2\text{Y}-\alpha 2$ and quenched at 77 K after 40 s. For each reaction, the residual $\text{Y}_{122}\bullet$ signal (blue) was subtracted from the composite reaction spectrum (black) to give a putative $\text{NH}_2\text{Y}_{413}\bullet$ (red). The inset gives an expanded view of the difference spectrum, which in the case of (A) and (B) corresponds to the putative $\text{NH}_2\text{Y}_{413}\bullet$.

FIGURE 5.

Reaction of wt $\beta 2$, CDP, ATP and (A) $Y_{413}NH_2Y-\alpha 2$ or (B) $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ monitored by SF UV-vis spectroscopy. The reaction was monitored for an increase at 325 nm ($\lambda_{\max}$ of $NH_2Y_{413}\bullet$) and a decrease at 410 nm ($\lambda_{\max}$ of $Y_{122}\bullet$). The reaction with $Y_{413}NH_2Y-\alpha 2$ (A) was complete within 2 seconds. The reaction with $Y_{731}F/Y_{413}NH_2Y-\alpha 2$ was monitored over 200 s (B), with the first 30 s expanded in the inset. Exponential fits to the data are indicated by solid black lines.

TABLE 1

Characterization of $\text{NH}_2\text{Y}^\bullet$ formation in $\alpha:\beta$ complexes with various on- and off-pathway mutations

$\beta:\alpha$ complex	% $\text{NH}_2\text{Y}^\bullet$ (EPR) ^a	% $\text{NH}_2\text{Y}^\bullet$ (SF UV-vis) ^a	first phase k_{obs} (s ⁻¹)	second phase k_{obs} (s ⁻¹)
wt- $\beta 2$:Y ₇₃₀ NH ₂ Y- $\alpha 2$ ^b	53	39	12.8	2.5
wt- $\beta 2$:Y ₇₃₁ NH ₂ Y- $\alpha 2$ ^b	45	35	19.2	2.7
Y ₃₅₆ F- $\beta 2$:Y ₇₃₀ NH ₂ Y- $\alpha 2$	<3% ^c	<2% ^c	--	--
Y ₃₅₆ F- $\beta 2$:Y ₇₃₁ NH ₂ Y- $\alpha 2$	<3%	<2%	--	--
wt- $\beta 2$:Y ₇₃₁ F/Y ₇₃₀ NH ₂ Y- $\alpha 2$	<3%	<2%	--	--
wt- $\beta 2$:Y ₄₁₃ NH ₂ Y- $\alpha 2$	12	8	4.8 ^d	0.5
wt- $\beta 2$:Y ₇₃₁ F/Y ₄₁₃ NH ₂ Y- $\alpha 2$	7	12	0.04	--
Y ₃₅₆ F- $\beta 2$:Y ₄₁₃ NH ₂ Y- $\alpha 2$	<3%	ND ^e	--	--

^aY₁₂₂• converted to $\text{NH}_2\text{Y}^\bullet$, as a percent of the starting [Y₁₂₂•].^bOriginally reported in reference 15.^cNo conversion detected within our lower limit of detection, approximated to be 3% of the total initial Y₁₂₂• by EPR and 2% by SF UV-vis spectroscopy.^dTaken as the average of the rate constants measured at 325 nm and 410 nm.^eNot determined.