Published in final edited form as:

Biochemistry. 2018 June 19; 57(24): 3402–3415. doi:10.1021/acs.biochem.8b00160.

Properties of Site-Specifically Incorporated 3-Aminotyrosine in Proteins to Study Redox-Active Tyrosines: *E. coli* Ribonucleotide Reductase as a Paradigm

Wankyu Lee^{†,*}, Müge Kasanmascheff^{§,@,*}, Michael Huynh^{||}, Anthony Quartararo[†], Cyrille Costentin^{||,▽}, Isabel Bejenke[§], Marina Bennati^{*,§}, Daniel G. Nocera^{*,||}, Cecilia Tommos^{*,||}, and JoAnne Stubbe^{*,†,‡}

[†]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

[‡]Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

[§]Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, Göttingen, 37077 Germany

*Corresponding Authors. marina.bennati@mpibpc.mpg.de, dnocera@fas.harvard.edu, tomomos@pennmedicine.upenn.edu, stubbe@mit.edu.

*These authors contributed equally.

ORCID

Wankyu Lee: 0000-0001-9048-6398

Marina Bennati: 0000-0001-6784-7845

Daniel G. Nocera: 0000-0001-5055-320X

Cecilia Tommos: 0000-0001-8095-8873

JoAnne Stubbe: 0000-0001-8076-4489

@Present Addresses

Müge Kasanmascheff: Department of Chemistry and Chemical Biology, Technical University of Dortmund, Otto-Hahn-Str. 6, 44227 Dortmund, Germany

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Figure S1. Thioredoxin- $\alpha_3\text{NH}_2\text{Y}$ expression profile as a function of $\text{NH}_2\text{Y-RS}$ induction OD_{600} (Figure S1); Tricine SDS PAGE analysis of purified $\alpha_3\text{NH}_2\text{Y}$ (Figure S2); LC-MS analysis of purified $\alpha_3\text{NH}_2\text{Y}$ (Figure S3); Spectroscopic measurements on $\alpha_3\text{NH}_2\text{Y}$; pH studies of $\alpha_3\text{NH}_2\text{Y}$ (Figure S4); CD characterization of $\alpha_3\text{NH}_2\text{Y}$ (Figure S5); $\alpha_3\text{NH}_2\text{Y}$ protein films display “split square-wave peaks” response (Figure S6); $\alpha_3\text{NH}_2\text{Y}$ protein films display “quasi-reversible maximum” response (Figure S7); DFT calculations on 2-aminophenol; Effect of NH_2 conformation on the 2- NH_2 -PheOH⁺/OH redox pair (Figure S8); Effect of NH_2 conformation on the 2- NH_2 -PheO⁻/O• redox pair (Figure S9); Synthesis of D₆-NH₂Y; 9 GHz EPR characterization of reacted His₆-D₆-NH₂Y_{731- α 2/wt- β 2/ATP/CDP; 9 GHz EPR spectra of His₆-D₆-NH₂Y_{731- α 2 reacted with wt- β 2, CDP and ATP (Figure S10); Spectral simulations of the multi frequency EPR data; The EPR spectra of D₆-NH₂Y_{731•} in H₂O recorded at 94 GHz at 80 K compared with H₆-NH₂Y_{731•} in D₂O (Figure S11); The EPR spectra of D₆-NH₂Y_{731•} with corresponding simulations for variation of the dihedral angle θ . (Figure S12); Syntheses of peptides P1 and P2; LC-ESI-MS on purified peptides P1, P2 and P3 (Figure S13), MS/MS analysis of peptides P1 and P2; MS/MS analysis of peptides P1 and P2 (Figure S14); LC-ESI-MS analysis of the trypsin-digested His₆-NH₂Y_{730- α 2} (Figure S15); MS quantitation of peptides P1 and P2 (Table S1); Inactivity of NH₂Y-RNRs and the issues that play a role in previously observed assays; The statistical distribution of heterodimeric of the His₆-NH₂Y_{730- α /wt- α (Figure S16)}}}

Author Contributions

All authors contributed to manuscript writing. W.L.^{†,*} prepared the $\alpha_3\text{NH}_2\text{Y}$ and NH₂Y-RNRs, prepared samples for EPR/ENDOR analysis, prepared samples for LC-MS analysis for Y contamination; M.K.^{§,@,*} prepared EPR/ENDOR samples, recorded and analyzed EPR data and contributed to analysis of ENDOR data; M.H.^{||} performed the DFT calculations on 2-aminophenol, A.Q.[†] designed and performed the LC-MS experiments; C.C. contributed to the analysis of electrochemical data; I.B.[§] performed ENDOR spectroscopy and analysis.

The authors declare no competing financial interest.

^{II}Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts, 02138 United States

^VUniversité Paris Diderot, Sorbonne Paris Cité, Laboratoire d'Electrochimie Moléculaire, Unité Mixte de Recherche Université – CNRS No 7591, Bâtiment Lavoisier, 15 rue Jean de Baïf, 75205 Paris Cedex 13, France

^{II}Department of Biochemistry and Biophysics, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, United States

Abstract

3-Aminotyrosine (NH₂Y) has been a useful probe to study the role of redox active tyrosines in enzymes. This report describes properties of NH₂Y of key importance for its application in mechanistic studies. By combining the tRNA/NH₂Y-RS suppression technology with a model protein tailored for amino acid redox studies (α_3 X, X = NH₂Y), the formal reduction potential of NH₂Y₃₂(O•/OH) ($E^{\circ'}$ = 395 ± 7 mV at pH 7.08 ± 0.05) could be determined using protein film voltammetry. We find that the $E^{\circ'}$ between NH₂Y₃₂(O•/OH) and Y₃₂(O•/OH) when measured under reversible conditions is ~300 – 400 mV larger than earlier estimates based on irreversible voltammograms obtained on aqueous NH₂Y and Y. We have also generated D₆-NH₂Y₇₃₁- α_2 of RNR, which when incubated with β_2 /CDP/ATP generates the D₆-NH₂Y₇₃₁•- α_2 / β_2 complex. By multi-frequency EPR (35, 94 and 263 GHz) and 34 GHz ¹H ENDOR spectroscopies, we determined the hyperfine coupling (hfc) constants of the amino protons that establishes RNH₂• planarity and thus minimal perturbation of the reduction potential by the protein environment. The amount of Y in the isolated NH₂Y-RNR incorporated by infidelity of the NH₂YRS/tRNA pair was determined by a generally useful LC-MS method. This information is essential to the usefulness of this NH₂Y probe to study any protein of interest and is employed to address our previously reported activity associated with NH₂Y-substituted RNRs.

Graphical abstract

INTRODUCTION

Site-specific incorporation of unnatural amino acids (UAAs) using the suppressor tRNA/RS technology has been used to study many enzymatic processes^{1–3} including redox reactions involving transiently formed tyrosyl radicals (Y•s).^{4–7} In the latter case, fluorotyrosines (F_nY, n = 2, 3)^{8–11} and 3-aminotyrosine (NH₂Y)^{12, 13} have served as particularly useful mechanistic probes. F_nYs display a broad range of pK_a's (6.4–7.8)^{10, 14–16} and formal reduction potentials $E^{\circ'}$ (–25 to +135 mV) relative to Y,^{15, 16} which are important to

assessing proton coupled electron transfer (PCET) mechanisms. Reduction potentials reported by DeFelippis *et al* in 1991¹⁷ suggested that NH₂Y could serve as a –190 to –290 mV “radical trap”^{12, 13} and provide evidence for Y• intermediates that are normally difficult to detect because of their high reactivity and short half-lives ($t_{1/2}$).⁴ In this paper we report new insights about NH₂Ys incorporated into proteins, important to understand if they are to be useful as general mechanistic probes of Y• intermediates.

Ribonucleotide reductases (RNRs) hold the record for the number of distinct Y•s involved in any enzyme-mediated transformations. They catalyze the reduction of nucleotides (CDP, UDP, ADP and GDP) to deoxynucleotides.^{18, 19} The *E. coli* RNR, a paradigm for the class Ia enzymes, is composed of two subunits, α 2 and β 2, which form the active α 2 β 2 complex upon substrate and allosteric effector binding.²⁰ This enzyme has a stable diferric-tyrosyl radical (Y₁₂₂•) cofactor in β 2 that generates a transient thiyl radical (C₄₃₉•) in α 2, which, in turn, initiates nucleotide reduction.^{21, 22} C₄₃₉- α 2 and its oxidant Y₁₂₂•- β 2 are proposed to be connected by a radical transfer (RT) pathway of >35 Å (Figure 1) involving Y₃₅₆ in β 2 and Y₇₃₁ and Y₇₃₀ in α 2.^{4, 23} Evidence to support this proposal was obtained by site-specific incorporation of NH₂Y at each of these sites. Incubation of each NH₂Y- α 2 (or NH₂Y- β 2) mutant with the appropriate second subunit, substrate, and effector resulted in formation of an aminotyrosyl radical (NH₂Y•), supporting the inclusion of these Ys in the RT pathway.^{12, 13} In addition, NH₂Y• formation has allowed the characterization of a tight α 2 β 2 complex²⁴ and provided insight into three distinct PCET steps within this pathway using multi-frequency and high-field electron paramagnetic resonance (HF EPR),²⁵ electron-nuclear double resonance (ENDOR),^{26–28} and pulsed electron-electron double resonance (PELDOR) spectroscopies.²⁹ Despite the incorporation of the NH₂Y thermodynamic sink into the >200 mV uphill RT pathway (Figure 2), the NH₂Y-RNRs retained steady state activities of 3 – 5% of the wild type (wt) reaction rate.^{12, 13}

In this report, suppressor tRNA/RS technology was used to incorporate NH₂Y¹² into an α -helical model protein known to support reversible amino acid oxidation-reduction (α_3 X).³⁰ Protein film voltammetry (PFV) on the resulting α_3 NH₂Y protein revealed a E° (pH 7.08 $\pm$ 0.05) of 395 $\pm$ 7 mV for NH₂Y₃₂(O•/OH), which is 587 mV lower than Y₃₂(O•/OH).^{16, 30} A 587 $\pm$ 7 mV deep thermodynamic trap makes it unlikely that the NH₂Y-RNRs will retain catalytic activity given the proposed thermodynamic landscape (Figure 2). One way to elevate E° (NH₂Y(O•/OH)) in the NH₂Y-RNRs is if the protein alters the ring planarity of the ground state or NH₂Y• structure, specifically by inducing a trigonal pyramidal NH₂ conformation. Here we report density functional theory (DFT) calculations on 2-aminophenol that suggest that this distortion could elevate E° by 190 – 390 mV. Our original multi-frequency EPR analysis of NH₂Y₇₃₀•-RNR²⁵ was interpreted to favor a pyramidal (RNH₂Y•) structure, whereas later quantum chemical calculations on a geometry optimized structure of NH₂Y₇₃₀•- α 2 and NH₂Y₇₃₁•- α 2 predicted an almost planar conformation of the trapped NH₂Y•s in the protein environment of α 2.^{26, 27} To obtain a direct measurement of the amino protons hyperfine coupling (hfc) and their orientations, we synthesized D₆-NH₂Y₇₃₁- α 2 and generated the D₆-NH₂Y₇₃₁•- α 2 in the presence of β 2, substrate, and effector. This strategy permits a distinction between the resonances of the amino protons and all resonances of the internal ¹Hs in NH₂Y•. Multi-frequency EPR and 34 GHz ¹H electron nuclear double resonance (ENDOR) spectroscopies on D₆-NH₂Y₇₃₁•-

$\alpha 2$ revealed that the EPR spectra are consistent with an almost planar $\text{NH}_2\text{Y}_{731}\bullet\text{-}\alpha 2$ structure, implying little perturbation on E° by the protein environment.

The new $E^\circ(\text{NH}_2\text{Y}_{32}(\text{O}\bullet/\text{OH}))$ value and the planar $\text{NH}_2\text{Y}_{731}\bullet$ structure, along with many previous studies on $\text{NH}_2\text{Y-RNRs}$,^{12, 13} establish that while NH_2Y functions as an excellent radical trap, its incorporation is unlikely to permit activity for $\text{NH}_2\text{Y-RNR}$ mutants. To explain our previously reported dNDP forming activity, we developed a LC-MS method to quantitate Y that might have been mis-incorporated and co-purified with $\text{NH}_2\text{Y-RNRs}$. Based on our analysis, 6% mis-incorporated Y is detected, which we propose is associated with the infidelity of evolved $\text{NH}_2\text{Y-tRNA/RS}$ pair and the growth conditions. Our studies provide important information for the general use of NH_2Y probe in enzymatic reactions that involve transient $\text{Y}\bullet\text{s}$ and provide an explanation for our previously reported activity of $\text{NH}_2\text{Y-RNRs}$.^{12, 13}

MATERIALS AND METHODS

Materials

D₇-L-tyrosine (D₇-Y, >98%) was purchased from Cambridge Isotope Laboratories. NH_2Y , ampicillin (Amp), chloramphenicol (Cm), 2XYT media, 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Life Technologies. Ultra pure urea was purchased from Nacalai Tesque, Kyoto, Japan. Fluorenylmethoxycarbonyl-protected 3-nitro-L-tyrosine (Fmoc-NO₂Y-OH) was purchased from Combi-blocks. Fmoc-L-arginine (Fmoc-R-OH) was purchased from Creo Salus. *N,N*-diisopropylcarbodiimide (DIC) was purchased from Chem-Impex. 4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) linker resin was purchased from ChemMatrix. O-(7-azabenzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (HATU) was purchased from Chem-Impex.

Protein production

$\text{His}_6\text{-NH}_2\text{Y}_{730}\text{-}\alpha 2$,¹³ wt- $\beta 2$,^{38, 39} $\alpha_3\text{Y}$, $\alpha_3\text{W}$ and $\alpha_3\text{F}^{40}$ were generated as previously described. Generation of $\text{His}_6\text{-D}_6\text{-NH}_2\text{Y}_{731}\text{-}\alpha 2$ followed published protocols with D₆- NH_2Y used in place of NH_2Y (yielding 1.6 mg pure $\text{His}_6\text{-D}_6\text{-NH}_2\text{Y}_{731}\text{-}\alpha 2$ /L of culture).²⁸ Expression and purification of $\alpha_3\text{NH}_2\text{Y}$ was conducted as follows: Expression vectors pET32b- $\alpha_3\text{TAG}_{32}$ (encoding the thioredoxin- His_6 -thrombinsite- $\alpha_3\text{X}$ sequence with the amber stop codon (TAG) at position 32 in the $\alpha_3\text{X}$ gene)^{15, 41} and pEVOL- $\text{NH}_2\text{Y-RS}$ (encoding the 3-aminotyrosine tRNA and aminoacyl-tRNA synthetase ($\text{NH}_2\text{Y-RS}$) genes)¹³ were co-transformed into *E. coli* BL21 (DE3) competent cells and grown overnight at 37 °C on LB/agar plates containing Amp (100 µg/mL) and Cm (35 µg/mL). A typical growth started with a single colony inoculated into a small culture (2 × 100 mL 2XYT media, containing 100 µg/mL Amp and 35 µg/mL Cm) and cultivated at 37 °C, 200 rpm until saturation (~16 h). The starting culture was diluted 50-fold into glycerol minimal media leucine (GMML;¹² 1 × M9 salts, 1% 0.05% (w/v) NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, (v/v) glycerol, and 0.3 mM L-leucine, 4 × 2 L) containing 100 µg/mL Amp, 35 µg/mL Cm, 1

mM NH₂Y, and 0.1 mM DTT. From the start of the growth, the GMML culture was also supplemented with 0.05% L-arabinose to induce over-expression of NH₂Y-RS. The early addition of L-arabinose was determined from the protein expression profile as a function of induction OD_{600 nm} (Figure S1). The cultures were grown at 37 °C, 200 rpm until reaching an OD_{600 nm} ~0.6 (~16 h), at which point thioredoxin- α_3 NH₂Y expression was induced by the addition of 1 mM IPTG. The cultures were grown at 37 °C, 200 rpm for an additional 4–6 h before the cells were harvested by centrifugation (13,000 $\times$ g). The expression of the full-length thioredoxin- α_3 NH₂Y was assessed by 15% acrylamide SDS-PAGE (Figure S2 and S3).

Voltammetry measurements

Differential pulse voltammetry (DPV), square-wave voltammetry (SWV) and cyclic voltammetry (CV) were performed using an Autolab PGSTAT12 potentiostat (Metrohm/Eco Chemie) equipped with a temperature-controlled, Faraday-cage protected micro-cell (Princeton Applied Research). The three-electrode configuration consisted of a pyrolytic graphite edge (PGE) working electrode (3 mm diameter; Bio-Logic, USA), a Ag/AgCl (3M KCl) reference electrode and a platinum wire counter electrode (both from Advanced Measurements). The counter electrode was filled with the electrolyte solution used for each specific measurement. α_3 NH₂Y and α_3 F (a redox-inactive Y₃₂F variant of α_3 Y)⁴² film samples were prepared by dissolving lyophilized protein in 20 mM KP_i, 75 mM KCl, pH 7.1 buffer to a final protein concentration of 15–20 μ M. The concentrations of α_3 NH₂Y and α_3 F were determined by absorption (ϵ_{290} 3290 $\pm$ 140 M⁻¹ cm⁻¹ at pH 7.04 $\pm$ 0.02; see SI) and by CD (titrating lyophilized α_3 F to a 222 nm ellipticity matching the 222 nm that of an α_3 W control) spectroscopy, respectively. PGE/protein film samples were prepared as previously described.¹⁶ Briefly, the tip of the freshly polished PGE electrode was submerged into the protein solution for 30 sec, the electrode was removed, its sides wiped to remove any residual protein solution, and then placed in the micro-cell containing only electrolyte solution. The pH in the protein sample and in the electrolyte buffer were matched and measured prior to and after data collection. Solution resistance was compensated for by using the Autolab positive feed-back iR compensation function. All potentials are given *versus* the normal hydrogen electrode (NHE). Buffers and protein samples were prepared using ultrapure chemicals (Sigma Aldrich) and data recorded under an argon atmosphere.

CV line shape analyses

The difference (E°) between $E^\circ_1(\text{NH}_2\text{Y}_{32}(\text{O}/\text{O}^\bullet))$ and $E^\circ_2(\text{NH}_2\text{Y}_{32}(\text{O}^\bullet/\text{OH}))$ (see Figure 3 for the α_3 NH₂Y redox states) was determined from CV peak current⁴³ and peak half-height width⁴⁴ analyses. For the former, the ratio of the peak current (i_p) to the normalized peak current ($i_{p, \text{norm}}$) is given by:⁴³

$$i_p/i_{p, \text{norm}} = i_p/(FSI^0Fv/RT) \quad \text{eq. 1}$$

where F is Faraday's constant (96,485 C/mol), R is the universal gas constant (8.314 J/K mol), T is the temperature (298 K), v is the CV scan rate (V/s), S is the electrode surface

area, and Γ^0 is the electrode surface coverage in moles per unit of surface area. Due to the two-electron stoichiometry of the wave, peak integration gives:

$$\int_0^\infty idt = \int_{-\infty}^\infty idE/v = A/v$$

and eq. 1 can be simplified to eq. 2:

$$i_p/i_{p,norm} = i_p/(AF/2RT) \quad \text{eq. 2}$$

i_p (peak height) and A (peak area) of the anodic and cathodic currents were obtained from background-corrected $\alpha_3\text{NH}_2\text{Y}$ cyclic voltammograms. Data processing and analyses were done using NOVA (Metrohm/Eco Chemie), PeakFit (Systat Software) and DigiElch (Gamry Instruments).

HF EPR and ^1H -ENDOR spectroscopy to determine the NH_2 conformation of $\text{D}_6\text{-NH}_2\text{Y}_{731}\alpha_2$

The samples for HF EPR and ENDOR measurements were prepared by mixing 215 μM $\text{His}_6\text{-D}_6\text{-NH}_2\text{Y}_{731}\alpha_2$, 215 μM wt- β_2 , 6 mM ATP and 2 mM CDP in protonated assay buffer (50 mM HEPES, 15 mM MgSO_4 , 1 mM EDTA, pH 7.6) and manually quenched in liquid isopentane after 10–25s. The 34 GHz ENDOR spectra were recorded on a Bruker E580 X/Q-band spectrometer equipped with an ENDOR resonator (Bruker EN 5107D2) and power-upgraded with a TWT amplifier of 170 W output power. The electron spin echo ($t_{\pi/2}-\tau-t_{\pi}-\tau$ – echo) was recorded with $t_{\pi/2}$ pulse length of 6 ns. Davies ENDOR ($t_{\pi}-\tau-t_{\pi}^{\text{RF}}-\tau-t_{\pi/2}-\tau-t_{\pi}-\tau$ – echo) was performed with $t_{\pi}=100$ ns for preparation, $t_{\pi}=12$ ns for detection and ^1H inversion pulse of $t_{\pi}^{\text{RF}}=20\mu\text{s}$ (250 W RF power, Amplifier Research). Samples of $\sim 10\mu\text{L}$ for 34 GHz EPR were quenched in 1.6 OD capillaries (Wilmad 222T-RB).

The 94 GHz pulsed EPR spectra were recorded on a Bruker E680 W-band spectrometer with 400 mW microwave output power (Bruker Power Upgrade 2). Samples for 94 GHz spectroscopy were 2 μL in 0.85 mm OD Suprasil capillaries. The electron spin echo ($\pi/2-\tau-\pi-\tau$ – echo) was recorded with $t_{\pi/2}$ pulse length of 20 ns. EPR measurements at 263 GHz were recorded on a Bruker Elexsys E780 quasi optical spectrometer using a single mode (TE_{011}) cylindrical resonator (Bruker BioSpin) as reported previously.^{28, 34} Maximum microwave power coupled to the resonator was ~ 50 mW. The electron spin echo was recorded with a $t_{\pi/2}$ pulse length of 26 ns. The EPR spectrum of $\text{Y}_{122}\bullet$ in wt- β_2 (250 μM in assay buffer) was used as a reference to calibrate the magnetic field values. Samples of ~ 50 nL for 263 GHz EPR were inserted in capillaries (0.33 OD, Vitrocom CV2033S/Q). Frozen samples were loaded into the resonator immersed in liquid nitrogen and then transferred into the pre-cooled EPR cryostat.

Simulation of multi-frequency EPR and ENDOR spectra

EPR spectra were simulated using the EasySpin-5.2.10 “pepper”-routine, which was run in MATLAB.⁴⁵ A combination of Gaussian (0.15 mT) and Lorentzian (0.15 mT) line broadening was used for all simulations. ENDOR spectra were simulated with EasySpin-5.2.12 using the ‘salt’-routine. To distinguish between a ‘flat/planar’ and a pyramidal NH₂ group we define θ , which describes the dihedral C-C-N-H angle between the Y ring plane and the plane defined by the C-N-H bonds (Figure 6A). This relates to the simulation parameters as follows. The direction of the NH bond coincides with the hfc tensor orientation of the *smallest* ¹H (NH) hfc, defined here as A_x ^{26, 27} (see Figure 6B), whereas the Y plane is spanned by the directions of the principal axes g_x and g_y of g (Figure 6B). Therefore, the angle θ is encoded in the *Euler* rotation $\mathbf{R}_{x,y,z}$ (where the indexes indicate the three rotation axes) around three consecutive angles (α , β , γ) connecting the $\mathbf{A}$ to the g principal axes system.⁴⁶ Euler angles are defined here in the right-handed coordinate systems for counter-clockwise rotations (magnetic resonance convention). As the A_z direction (i.e. the direction of the largest hfc component) was predicted in the C-N-H plane (Figure 6B),²⁷ the angle θ is encoded in the first Euler rotation $\mathbf{R}_z(\alpha)$ or $\theta = |\alpha| - 90^\circ$.

LC-ESI-MS quantitation of the NH₂Y:Y ratio in trypsin-digested NH₂Y₇₃₀- α 2

The sequence of the peptide from the trypsin-digested wt- α 2 was predicted with MS-digest (www.prospector.ucsf.edu). The Y-containing peptide P1 (H₂N-TLYYQNTR-COOH) and the NH₂Y-containing peptide P2 (H₂N-TL(NH₂Y)YQNTR-COOH) were synthesized via automated flow peptide synthesis as described in the SI.⁴⁷ To quantitate the percentage of Y mis-incorporation in trypsin-digested His₆-NH₂Y₇₃₀- α 2, we needed to correct for the differing ionization tendencies for each peptide when present in a mixture of peptides. Thus, we sought to establish a relative ratio of the ion intensities from injecting an equal amount (1:1 w/w ratio) of each peptide and constructing a peptide standard curve with the expectation that at different injection amounts, the intensity ratio would remain constant in a given range. Peptide stock solutions were prepared in water/0.1% TFA, and the concentrations were determined with predicted extinction coefficients for P1 ($\epsilon_{280\text{ nm}} = 2880\text{ M}^{-1}\text{ cm}^{-1}$) and P2 ($\epsilon_{280\text{ nm}} = 3340\text{ M}^{-1}\text{ cm}^{-1}$). The P1 $\epsilon_{280\text{ nm}}$ was predicted from peptide sequence, using the previously determined $\epsilon_{280\text{ nm}} = 1440\text{ M}^{-1}\text{ cm}^{-1}$ for each Y in H₂O/HCl, pH 1.^{48, 49} The P2 $\epsilon_{280\text{ nm}}$ was predicted from the peptide sequence where the $\epsilon_{280\text{ nm}}$ of $1900\text{ M}^{-1}\text{ cm}^{-1}$ for NH₂Y was previously determined in H₂O/HCl, pH 1.²⁵ The peptide standard curve was constructed by analyzing 0.1 ng to 10 ng of each peptide by ESI-MS.

The MS experiments were performed using an Agilent 6550 ESI-MS qTOF equipped with a Jupiter C4 reverse phase column (Phenomenex, 50 mm $\times$ 1 mm, 5 μ m particle size, 300 Å pore size). The gradient consisted of two solvents: solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA. Solvent B was linearly increased from 1 to 61% over 16 min at a flow rate of 0.1 mL/min. Prior to the next run, the column was equilibrated with 1% solvent B for 5 min at the same flow rate. In between every sample, a blank sample of no volume was injected to ensure no carryover from the previous injection. The reverse phase column was coupled to the electrospray ion source (Agilent 6550). The ions were introduced to a quadrupole time-of-flight (Q-TOF) mass analyzer operating in the positive ion mode. The MS spectra were obtained by scanning from 300 to 1700 m/z , with a resolution of

30,000. An internal standard (HP-0921, Agilent, theoretical positive ion mass = 922.0097 m/z) was used to calibrate ion masses. From the total ion chromatogram (TIC), the +2 and +3 ions corresponding to peptides P1 and P2 were extracted with the MassHunter workstation (Agilent). The peak extraction was performed with a m/z window of ± 0.01 mass units, which is within the resolution of the MS instrument. To determine relative peak area ratios for the standard curve, the extracted peaks were integrated with the MassHunter software.

The trypsin digestion procedure for His₆-NH₂Y₇₃₀- α 2 was optimized to minimize NH₂Y oxidation. The protein (40 μ M) was denatured and reduced in 40 μ L trypsin digestion buffer (50 mM NH₄HCO₃ pH 7) containing 8 M urea and 5 mM TCEP for 1 h at 37 °C. Trypsin digestion buffer with TCEP to 5 mM was added to dilute the urea to 2 M and the denatured protein was incubated with Trypsin Gold (1:50 w/w trypsin: protein) for 3 h at 37 °C. The resulting peptide mixture was analyzed by LC-ESI-MS by the method described for the standard curve determination. After the peaks for the +2 and +3 ions for P1 and P2 were extracted, relative amounts of each peptide were determined by normalizing the relative ratios of peak areas in each sample to those from the standard curve. From the trypsin-digested peptide mixture, eight different amounts were injected to provide multiple pairs of signal intensities to reference. The reported errors were calculated as standard deviations from replicate samples at each injection amount.

RESULTS

To determine the thermodynamic landscape in the NH₂Y-RNRs (Figure 2), we initially sought to reinvestigate the thermodynamic redox properties of NH₂Y. Our efforts using cyclic voltammetry on NH₂Y (aq) resulted in quasi-reversible voltammograms involving more than a single charge transfer and from which no detailed thermodynamic information could be extracted (data not shown). Quasi/irreversible voltammograms are typical of phenolic compounds in solution and reflect the short $t_{1/2}$ of the reactive radical state.^{30, 42, 50} The α_3 X family of well-structured model proteins was developed to address this issue.^{40–42, 51, 52} These proteins share a common three-helix bundle (α_3) with a redox site at position 32 (X₃₂) in the protein interior.³⁷ The α_3 scaffold increases the radical $t_{1/2}$ dramatically ($\times 10^4$ for Y₃₂• and W₃₂• relative to Y• (aq) and W• (aq))^{42, 52} and has allowed reversible voltammograms and E° values to be obtained on Y₃₂, W₃₂ and a number of tyrosine analogs.^{15, 16, 30, 51, 52}

Voltammetry analysis of α_3 NH₂Y

Purification and thrombin digestion of thioredoxin- α_3 X followed the standard α_3 X protein purification protocol,⁴⁰ with two modifications to minimize NH₂Y oxidation: 10 mM β -mercaptoethanol was added to the lysis buffer and Ni²⁺ column elution buffer and the cleavage reaction was carried out in an anaerobic glove box. The purity of α_3 NH₂Y was assessed by 15% tricine SDS-PAGE analysis and LC-ESI-MS (Figures S2 and S3). A typical yield was ~0.4 mg pure α_3 NH₂Y/L of culture.

NH₂Y₃₂ was expected to be a 2e⁻ redox system, as reported for 2-aminophenol^{53, 54} and illustrated in Figure 3. The degree of difficulty extracting $E^{\circ'}_1$ and $E^{\circ'}_2$ from the α_3 NH₂Y

voltammogram depends on their relative order and separation (i.e. the sign and magnitude of $E^{\circ'} = E^{\circ'}_1 - E^{\circ'}_2$, *vide infra*). Moreover, multiple redox couples may contribute, e.g. $(\text{NH}_3)^+\text{Y}(\text{O}\bullet/\text{OH})$, $\text{NH}_2\text{Y}(\text{O}\bullet/\text{OH})$ or $\text{NH}_2\text{Y}(\text{O}\bullet/\text{O}^-)$, depending on the experimental pH and the pK_a 's of the ring NH_2 and OH groups.^{55, 56} To reduce the complexity of the analysis, a series of optical and voltammetry studies were conducted to establish conditions for which the $\alpha_3\text{NH}_2\text{Y}$ voltammogram was shown to represent a reversible, surface-confined $2e^-$ redox process involving the initial and final redox states shown in Figure 3. Multiple cyclic voltammograms were then collected at these conditions, background-subtraction routines evaluated, and the positions and line shapes of the processed traces analyzed to determine $E^{\circ'}_1(\text{NH}_2\text{Y}_{32}(\text{O/O}\bullet))$ and $E^{\circ'}_2(\text{NH}_2\text{Y}_{32}(\text{O}\bullet/\text{OH}))$. These studies are summarized below.

Defining experimental settings for CV line shape analyses

The first step was to determine the pH range for which the protonation state of reduced amino- Y_{32} is well defined and that the global protein properties of $\alpha_3\text{NH}_2\text{Y}$ are similar to those of the structurally well-characterized $\alpha_3\text{Y}$ and $\alpha_3\text{W}$ proteins.^{37, 40–42, 52} An absorption pH titration showed that the reduced protein is in the $\text{NH}_2\text{Y}_{32}\text{--OH}$ state²⁵ between pH 4.8 – 10 and that the OH remains fully protonated (Figure S4A).²⁵ Circular dichroism and fluorescence studies demonstrated that $\alpha_3\text{NH}_2\text{Y}$ displays an α -helical content and global stability typical of the $\alpha_3\text{X}$ proteins and that only minor changes occur in the α -helical content between pH 4.7–8.5 (Figure S5). The CD measurements were conducted in the presence of TCEP to ensure that $\alpha_3\text{NH}_2\text{Y}$ was fully reduced. TCEP absorption prevented CD spectra from being collected above pH 8.5. Nonetheless, a pH range of ~5 to 8.5 was broad enough to show that the $\alpha_3\text{NH}_2\text{Y}$ peak potential changes by $57 \text{ mV} \pm 1/\text{pH}$ unit (Figure S4B). Together these results demonstrate that voltammetry conducted at neutral pH involves only one reduced state ($\text{NH}_2\text{Y}_{32}\text{--OH}$) and a stoichiometric $ne^-/n\text{H}^+$ redox process, consistent with the redox states shown in Figure 3.

From this point on, PFV was employed for all measurements. Protein films were prepared as described previously¹⁶ and investigated by SWV, which provides well-established diagnostics for surface-confined electrode reactions.^{57, 58} The $\alpha_3\text{NH}_2\text{Y}/\text{PGE}$ protein film system displayed the “split SW peak” (Figure S6) and “quasi-reversible maximum” (Figures S7A and S7B) response consistent with a surface electrode mechanism. A combination of protein film CV and SWV data unambiguously assigned the observed Faradaic current to the NH_2Y_{32} residue and showed that this current represents a $2e^-$ redox process. The former is clear by comparing cyclic voltammograms of $\alpha_3\text{NH}_2\text{Y}$ (Figure 4A, red trace) and the redox inactive $\alpha_3\text{F}$ protein⁴² (Figure 4A, gray trace). Additionally, earlier studies have shown that the $1e^-$ $\alpha_3\text{X}$ proteins have a very poor CV response.⁴⁰ Thus the prominent current of $\alpha_3\text{NH}_2\text{Y}$, relative to the barely detectable CV response from the $1e^-$ $\alpha_3\text{X}$ proteins, is consistent with the expected $2e^-$ electrode mechanism for $\alpha_3\text{NH}_2\text{Y}$.^{53, 54} Likewise, the SW net current of $\alpha_3\text{NH}_2\text{Y}$ is almost twice the height relative to the SW net currents of the $1e^-$ $\alpha_3\text{Y}$ and $2\text{MP-}\alpha_3\text{C}$ proteins (Figure S7C). Finally, CV conducted using a scan rate of 250 – 500 mV/s generated fully reversible voltammograms (Figure 4C, *vide infra*). Thus, cyclic voltammograms collected on $\alpha_3\text{NH}_2\text{Y}/\text{PGE}$ films at neutral pH and using a scan rate of 250 – 500 mV/s represent a reversible, surface-confined $2e^-$ process with $\text{NH}_2\text{Y}_{32}(\text{O/O}\bullet)$ and $\text{NH}_2\text{Y}_{32}(\text{O}\bullet/\text{OH})$ as the dominant redox pairs.

CV line shape analyses

Our main goal was to determine $E^{\circ'}_2(\text{NH}_2\text{Y}_{32}(\text{O}\cdot/\text{OH}))$ (Figure 3) and several scenarios were possible as illustrated in Figure 5A. When $E^{\circ'}_1 > E^{\circ'}_2$, and the difference between the two ($E^{\circ'} = E^{\circ'}_1 - E^{\circ'}_2$) is > 60 mV, the voltammogram displays two resolved oxidation-reduction waves and extracting $E^{\circ'}_1$ and $E^{\circ'}_2$ is straightforward (Figure 5A, red trace). When $E^{\circ'}_1 > E^{\circ'}_2$ and the difference between the two is < 50 mV, the two waves begin to merge into a single wave (Figure 5A, blue and orange traces). Here the peak height and half-height width ($W_{1/2}$) varies as a function of $E^{\circ'}$. As $E^{\circ'}$ becomes negative, the single wave line shape becomes increasingly less sensitive to $E^{\circ'}$. When $E^{\circ'} < -200$ mV (Figure 5A, black trace), $E^{\circ'}_1$ and $E^{\circ'}_2$ can no longer be distinguished.

$\alpha_3\text{NH}_2\text{Y}$ gives rise to a single wave voltammogram (Figure 4A) for which the average of the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials were taken to equal $E^{\circ'}(2e^-)$ and $(E^{\circ'}_1 + E^{\circ'}_2)/2$. Peak current⁴³ and peak width⁴⁴ analyses were conducted to extract $E^{\circ'}$ (Figures 5B and 5C) and when combined with $E^{\circ'}(2e^-)$, the absolute values of $E^{\circ'}_1(\text{NH}_2\text{Y}_{32}(\text{O}/\text{O}\cdot))$ and $E^{\circ'}_2(\text{NH}_2\text{Y}_{32}(\text{O}\cdot/\text{OH}))$ could be determined. This was done as follows. Protein film staircase cyclic voltammograms were collected on $\alpha_3\text{NH}_2\text{Y}$ and $\alpha_3\text{F}$ using a scan rate of 250, 300, 350, 400, 450 and 500 mV/s. Representative raw and $\alpha_3\text{NH}_2\text{Y}$ – $\alpha_3\text{F}$ difference voltammograms are shown in Figure 4A. Both raw and difference traces were analyzed using two separate methods for background correction. Peak current information was obtained using a linear baseline function in NOVA and by using PeakFit. The latter approach is demonstrated in Figure 4B. Peak positions, heights, areas, and $W_{1/2}$ values were averaged over 84 anodic and cathodic traces with the following average results: electrode surface coverage (Γ^0) $16\text{--}19 \pm 2$ pmol/cm², $E_{\text{peak}} = (E_{\text{pa}} - E_{\text{pc}})$ $33\text{--}36 \pm 3$ mV, $i_{\text{pa}}/i_{\text{pc}}$ $1.00\text{--}1.03 \pm 0.02$, $i_{\text{p}}/i_{\text{p,norm}}$ $0.57\text{--}0.60 \pm 0.02$, and $W_{1/2}$ $80\text{--}85 \pm 3$ mV. These results show that there is no significant difference in the extracted line shape information when using raw vs difference voltammograms or when using the NOVA vs PeakFit fitting routines. Importantly, the independence of E_{peak} and $i_{\text{pa}}/i_{\text{pc}}$ on the scan rate is consistent with a reversible process with no influence from electron transfer kinetics. A finite peak splitting of $33\text{--}36 \pm 3$ mV was observed in contrast to theory, which predicts a E_{peak} of zero for a Nernstian surface electrode mechanism. This non-ideal behavior is commonly observed for protein films;

E_{peak} of zero is rarely, if ever, observed.⁴⁴ The independence of Γ^0 on the scan rate and the independence of the peak separation in the range of 250 to 500 mV/s (Figure 4C) indicates that the peak separation at low scan rate is not due to interference of electron diffusion within the film. The $i_{\text{p}}/i_{\text{p,norm}}$ was calculated from the height and area of the anodic and cathodic peak currents (eq. 2 in Materials and Methods). Comparing the $\alpha_3\text{NH}_2\text{Y}$ $i_{\text{p}}/i_{\text{p,norm}}$ of $0.57\text{--}0.60 \pm 0.02$ and a $W_{1/2}$ of $80\text{--}85 \pm 3$ mV values against $i_{\text{p}}/i_{\text{p,norm}}$ vs $E^{\circ'}$ (Figure 5B)⁴³ and $W_{1/2}$ vs $E^{\circ'}$ ⁴⁴ (Figure 5C), resulted in a $E^{\circ'}$ of 23 ± 5 mV.

Protein film linear cyclic voltammograms were also collected on $\alpha_3\text{NH}_2\text{Y}$ (250 – 500 mV/s; Figure 4C) and gave the following results: Γ^0 9 ± 1 pmol/cm², E_{peak} 43 ± 2 mV, $i_{\text{pa}}/i_{\text{pc}}$ 0.95 ± 0.03 , $i_{\text{p}}/i_{\text{p,norm}}$ 0.51 ± 0.02 , and $W_{1/2}$ 93 ± 4 mV. Comparing the linear CV $i_{\text{pa}}/i_{\text{pc}}$ and $W_{1/2}$ values to the working curves (Figures 5B and 5C)⁴³ predicted a $E^{\circ'}$ ~ 10 mV larger relative to the staircase CV result. Combining all data, the average $E^{\circ'} = (E^{\circ'}_1(\text{NH}_2\text{Y}_{32}(\text{O}/\text{O}\cdot)) - E^{\circ'}_2(\text{NH}_2\text{Y}_{32}(\text{O}\cdot/\text{OH})))$ is 26 ± 7 mV. With an $E^{\circ'}(2e^-; \text{pH } 7.08 \pm 0.05)$ of 408 ± 2 mV, E

$^{\circ}{}'_1(\text{NH}_2\text{Y}_{32}(\text{O}/\text{O}^{\bullet}))$ and $E^{\circ}{}'_2(\text{NH}_2\text{Y}_{32}(\text{O}^{\bullet}/\text{OH}))$ were determined to be 421 ± 7 mV and 395 ± 7 mV, respectively. The previously determined $E^{\circ}{}'_1$ of $\text{Y}_{32}(\text{O}^{\bullet}/\text{OH})$ at pH 7.08 of 982 ± 3 mV,^{16, 30} results in a $E^{\circ}{}'_1$ between NH_2Y_{32} and Y_{32} of -587 ± 8 mV.

The NH_2 conformation of $\text{D}_6\text{-NH}_2\text{Y}_{731}^{\bullet}$

With the one-electron NH_2Y reduction potential determined, the NH_2Y -RNRs are expected to be inactive as the 587 mV sink in the RT pathway would be too great to overcome thermodynamically. These NH_2Y -RNR mutants, however, were previously determined to be active.^{12, 13} Protein environments can alter reduction potentials substantially.⁵ A rationalization of NH_2Y -RNRs activities, therefore, could be based on our previous spectroscopic analysis of $\text{NH}_2\text{Y}_{730}^{\bullet}$ and the ambiguity associated with the $\text{NH}_2^{\bullet}$ conformation^{59, 60} based on methods available at the time.^{25–27} We thus undertook DFT calculations and developed a new method to directly determine the hfcs associated with the two $\text{NH}_2^{\bullet}$ protons, a key indicator of geometric distortion.

DFT calculations on 2-aminophenol were employed to distinguish between relative reduction potentials of flat ($\theta \approx 0^\circ$, Figure 6) vs. trigonal pyramidal ($\theta > 30\text{--}50^\circ$, Figure 6) NH_2 conformations based on our previous analyses.^{25–27} These results are described in Figures S8 and S9 and demonstrate that the trigonal pyramidal conformation can potentially increase the reduction potential by 190 – 390 mV.

Previously a trigonal pyramidal NH_2 structure was proposed based on the simulations of ^1H hfcs detected via the EPR spectra of [^{14}N] and [^{15}N] labeled $\text{NH}_2\text{Y}_{730}^{\bullet}$ in D_2O and H_2O buffer.²⁵ At the time these studies were conducted, the ^1H hfc of the exchangeable amino protons could not be directly determined because of the large, interfering signals of all other tyrosyl protons and hydrogen bonds. However, in our subsequent studies, DFT calculations on large cluster models that were generated from the crystal structure of $\text{NH}_2\text{Y}_{730}\text{-}\alpha 2$ (PDB 2XO4) suggested an almost planar NH_2 conformation in $\text{NH}_2\text{Y}_{730}^{\bullet}$ and $\text{NH}_2\text{Y}_{731}^{\bullet}$.^{26, 27} Thus, we decided to re-examine the NH_2 conformation in $\text{NH}_2\text{Y}_{731}^{\bullet}$ -RNR.

Exchangeable hydrogen-bonded protons with large hfcs are usually detected via EPR spectroscopy on D_2O samples. However, we showed in previous ENDOR studies that the ^2H spectra of the $\text{NH}_2\text{Y}^{\bullet}$ contained overlapping contributions from external hydrogen bonds and amino deuterons.^{26, 27} Combined with the fact that standard Mims ^2H -ENDOR sequence produces blind spots in the ENDOR line-shape, our previous studies prevented an unambiguous analysis of the NH_2 tensors. To simplify the EPR and ENDOR spectra and assign the structure of the NH_2 , we used a new labeling strategy in which the non-exchangeable protons of NH_2Y were replaced with deuterons ($\text{D}_6\text{-NH}_2\text{Y}$). The same approach was recently used for the ^1H -ENDOR study of $\text{D}_7\text{-Y}_{356}^{\bullet}$.³⁴ The $\text{D}_6\text{-NH}_2\text{Y}$ was site-specifically inserted and investigated at Y_{731} , as our previous studies²⁷ showed only one perpendicular hydrogen bond (H-bond) contributes to $\text{NH}_2\text{Y}_{731}^{\bullet}$ as compared to two perpendicular H bonds at residue $\text{NH}_2\text{Y}_{730}^{\bullet}$. These H bonds were shown to be associated with adjacent pathway residues.²⁷ We first established that $\text{D}_6\text{-NH}_2\text{Y}_{731}\text{-}\alpha 2$ in the presence of $\beta 2$, ATP and CDP could generate sufficient $\text{D}_6\text{-NH}_2\text{Y}_{731}^{\bullet}$ (18% relative to the initial $\text{Y}_{122}^{\bullet}$ as described in Figure S10, SI, and methods) for spectroscopic analysis.

The $D_6\text{-NH}_2Y_{731}\bullet$ was next generated in H_2O and analysed via multi-frequency EPR and 34 GHz 1H -ENDOR spectroscopy. As described in our previous HF EPR studies,^{25–28, 34} the spectrum of the trapped radical can be separated from that of stable $Y_{122}\bullet$ by pulsed EPR at 80 K. The recorded 34, 94 and 263 GHz EPR spectra are shown in Figures S11 and S12, along with simulations for a planar NH_2 structure. The large hfcs of the two amino protons and the nitrogen nucleus dominate the $D_6\text{-NH}_2Y_{731}\bullet$ EPR spectrum at 34 GHz (Figure S12). Conversely, 94 and 263 GHz EPR spectra are dominated by g and hyperfine A tensors.⁶¹ The g values of $D_6\text{-NH}_2Y_{731}\bullet$ (2.0051, 2.0040, 2.0022) are best resolved at 263 GHz and are consistent with the values from our previous ND_2Y studies.^{25–28} Comparison of 94 GHz EPR spectra of $H_6\text{-NH}_2Y\bullet$ in D_2O and $D_6\text{-NH}_2Y\bullet$ in H_2O (Figure S11) clearly reveals the advantages of our deuteration approach. Use of $D_6\text{-NH}_2Y\bullet$ in H_2O considerably simplifies the EPR spectra due to the absence of the two $C\beta^1H$ hf splitting and subtle hf structure from the amino protons becomes visible.

The starting simulation parameters were taken from the quantum chemical calculations of $NH_2Y_{731}\bullet$ (model 3 in Ref 27) with a planar ($\theta \approx 0^\circ$) NH_2 structure (Figure 6). First, we checked the validity of the predicted hfc principal axes value $A_{x,y,z}$ by recording and simulating a 34-GHz 1H -Davies ENDOR spectrum of $D_6\text{-NH}_2Y\bullet$ in H_2O (Figure 7). We note that the absence of orientation selection in these 34 GHz 1H ENDOR spectra permits examination of the $A_{x,y,z}$ values *independently* from the g and A tensor orientations (i.e. their interconnecting Euler angles). We find that this 1H ENDOR spectrum of the exchangeable protons (two $^1H\text{-NH}$ and one H bond) is consistent with the predicted DFT values used in the simulation (Figure 7 and Table 1) that is apparent by the matched resonances at the canonical points (maxima and edges). Importantly, the intermediate A_y values of both $^1H\text{-NH}$ are best resolved ($A_y \approx 17$ MHz). The ENDOR spectrum also shows some broadening around A_z , consistent with our previous observation that the $^1H\text{-NH}$ resonances are sensitive to the H bond environment and heterogeneities.²⁷

To simulate the multi-frequency EPR spectra of $D_6\text{-NH}_2Y\bullet$ we employed the validated $A_{x,y,z}$ values and found that the simulation with the planar NH_2 structure (Figures 6 and S12) readily satisfied the observed hf pattern in the EPR spectra after an adjustment of $\sim 10\%$ of the A_z values for the $^1H\text{-NH}$ (see Table 1). We note that the complete parameter space of the EPR simulation is quite large (36 parameters, first six columns in Table 1 in addition to line width and unknown heterogeneities), which prevented a rigorous fitting of the data. The effect of the tilt angle θ was tested by assuming $\theta_1 = \theta_2 = \theta$ and varying its value between 0° and 50° . For simplicity we kept all $A_{x,y,z}$ values constant, as our DFT calculation on the aminophenol radical predicted only minor change ($\sim 10\%$) of the isotropic $^1H\text{-NH}$ hfcs by variation of θ . In Figure 6 we display representative simulations of the 94 GHz spectra, which are most sensitive to the angle θ . Further simulations and residual analysis for variation of θ between 0° and 50° in 10° steps are reported in Figure S12. The analysis of residuals for 94 GHz data demonstrated that the simulations having $\theta > 20^\circ$ significantly deviate from the experimental spectrum. For θ increasing between 0° and 50° the simulations show first a gradual disappearing of the hyperfine structure in the center of the spectrum (Figure 6, $\theta = 25^\circ$) and then the appearance of a new hf pattern (Figure 6, $\theta = 50^\circ$), which is different from the experiment. Indeed, in a planar conformation, the A tensor

principal axes of the amino protons are almost collinear with the **g** principal axes (Figure 6B) and it is expected⁶¹ that this collinearity gives rise to the sharp hf splitting in the HF-EPR spectra. These sharp features are visible in the spectra of D₆-NH₂Y₇₃₁• but were not discernible in the spectra of H₆-NH₂Y₇₃₁•, reported in ref. 25 (see Figure S11), due to the broadening of the internal Y protons. We conclude that our multi-frequency EPR spectra are consistent with an planar NH₂ conformation or $\theta = (0 \pm 20)^\circ$.

The NH₂Y:Y ratio in NH₂Y₇₃₀- α 2

The PFV and spectroscopic results described above together require NH₂Y-RNRs to be inactive. Given our previous reports on their activity^{12, 13}, we developed a method to quantitatively assess the amount of wt- α 2 contaminating NH₂Y₇₃₀- α 2 to account for our observations. Wt- α 2 contamination can result from *E. coli* used for expression or from mis-incorporation of Y associated with the specificity of the evolved NH₂Y-RS.¹³ We believe that wt- α 2 has been removed as we use a His₆-NH₂Y₇₃₀- α 2 construct and Ni²⁺-affinity column for purification. The fidelity of the NH₂Y-RS was previously determined by MALDI-TOF MS on the NH₂Y incorporated into the Z-domain of protein A; no evidence for Y incorporation was apparent.¹² However, the NH₂Y-RS fidelity may differ depending on gene sequence and on the ratio of Y/NH₂Y in the growth media.

The determination of the percentage of mis-incorporated Y in His₆-NH₂Y₇₃₀- α 2 was carried out on trypsin-digested protein, because the mass difference of 14 Da between Y and NH₂Y cannot be resolved with the whole protein by MS. This process required extensive optimization due to sensitivity of NH₂Y to oxidation. In the final procedure the reaction mixture contained 5 mM TCEP, trypsin (1:50 w/w protease/protein) and only a 3 h incubation. The success of the digestion was verified by LC-ESI-MS/MS (97% protein coverage); details are described in Methods and SI.

LC-ESI-MS was used to quantitate the relative ratios of peak areas for the predicted trypsin-digested peptides, P1 (NH₂TL(NH₂Y)YQNTRCO₂H) and P2 (NH₂TL(Y)YQNTRCO₂H). Initially P1 and P2 were synthesized using automated flow peptide synthesis (SI and Figure S13)⁴⁷, purified to homogeneity by C18 reverse phase HPLC and analyzed by LC-ESI-MS (Figure S13). P1 predominantly ionized in the +3 charge state (358.52 *m/z*), whereas the P2 preferred the +2 charge state (529.77 *m/z*). Importantly, P1 remained reduced (Figure S14) during this analysis. To account for the differences in ionization efficiencies for each peptide, a 1:1 (w/w) ratio of P1 and P2 was prepared and analyzed over a 0.1 ng to 10 ng range. Standard curves were constructed for both +2 and +3 charge states for P1 and P2 as shown in Figure 8. These curves established a relative ratio between P1 and P2 over a range of amounts for MS analysis within the linear dynamic range of the instrument. As expected, their relative ratio remained constant over the selected range, which also then served as the target for injection amounts of trypsin-digested sample.

With the relative ratios now established by the standard curves, the relative abundances for P1 and P2 in the trypsin-digested His₆-NH₂Y₇₃₀- α 2 were characterized. P1 and P2 were assumed to have equal trypsin cleavage efficiencies because the Y₇₃₀ or NH₂Y₇₃₀ is five residues from the peptide cleavage site, R₇₃₅. The LC-ESI-MS analysis of the trypsin-digested His₆-NH₂Y₇₃₀- α 2 yielded the total ion chromatogram (TIC) displayed in Figure

S15. P1 and P2 were identified by their preferred ionization states using ion extraction with the MassHunter software (Agilent). The peak for P1 eluted at a retention time of 5.9 min and for P2 at 6.8 min (Figure S15). To determine the relative amounts of P1 and P2, the relative ratios of the peak areas were normalized to those obtained from the standard curve. The peak areas and calculated peptide quantities for eight amounts are summarized in Table S1. From these results, P2 was determined to be $6.1 \pm 0.5\%$ relative to total peptide concentration, defined as the sum of the P1 and P2 concentrations. Each measurement is depicted on the standard curve in Figures 8A and 8B with green and red dashed lines indicating P1 and P2 amounts, respectively. This experiment was reproduced with a His₆-NH₂Y₇₃₀-α2 isolated by a different researcher,¹³ yielding $6.9 \pm 0.4\%$ P2 relative to the total peptide concentration. These results provided strong evidence that sufficient mis-incorporation of Y by the NH₂Y-RS into NH₂Y-RNR can explain the observed dCDP formation in the steady state activity assays.

DISCUSSION

NH₂Y oxidation

NH₂Y is a useful mechanistic probe for enzymes utilizing redox-active Y residues in catalytic and RT processes.^{5, 62–67} With a E° (pH 7.08 $\pm$ 0.05; NH₂Y₃₂ – Y₃₂) of -587 ± 8 mV, NH₂Y is predicted to form a deep thermodynamic trap when site-specifically incorporated at a redox-active Y site. Additionally, the EPR *g* tensor of the resulting NH₂Y• is a probe of its electrostatic environment. NH₂Y can also undergo 2e[−] oxidation in aqueous solution under mild conditions and has been used in chemo-selective bio-conjugation to modify proteins of interest with anilines site-selectively incorporated.^{68–70} Despite its utility, E° (1e[−]) values for NH₂Y have remained difficult to determine due to irreversibility associated with competing reactions during the measurements and the rapid loss of the second electron.

In our previous analyses on the thermodynamics of RT in RNR (Figure 2),

$E(\text{NH}_2\text{Y}(\text{O}^\bullet/\text{OH}) - \text{Y}(\text{O}^\bullet/\text{OH}))$ predictions were based on studies on aqueous NH₂Y¹⁷ and Y.^{4, 37} DeFelippis *et al* reported a pH 7 value of 640 mV using voltammetry peak potentials corrected for chemical reactivity of the oxidized product(s).¹⁷ With reversibility issues for both NH₂Y (aq) and Y (aq) and the e[−] stoichiometry of the former an open question, we revisited the question of E° (NH₂Y(O•/OH)) and how it compares to E° (Y(O•/OH)).

To study NH₂Y under fully reversible conditions and obtain a firm E° (NH₂Y(O•/OH) – Y(O•/OH)) value, we turned to the α₃X system and PFV.³⁰ Voltammetry characterization of the 2e[−] α₃NH₂Y redox system differs relative to the previously studied 1e[−] α₃Y,^{16, 30} 2-mercaptophenol-α₃C,⁵¹ the α₃F_nYs,^{15, 16} and α₃W⁵² proteins. The combination of a less oxidizing potential range and a 2e[−] current, yielded protein film cyclic voltammograms of excellent quality (e.g. Figure 4). We found that $E^{\circ\prime}_1(\text{NH}_2\text{Y}_{32}(\text{O}/\text{O}^\bullet))$ and $E^{\circ\prime}_2(\text{NH}_2\text{Y}_{32}(\text{O}^\bullet/\text{OH}))$ are of “normal order” (i.e. $E^{\circ\prime}_1 > E^{\circ\prime}_2$) and in the E° region in which they can be accurately determined (Figure 5) are 421 ± 7 mV and 395 ± 7 mV, respectively. This is the first determination of these redox couples. NH₂Y₃₂(O•/OH) is 587 ± 8 mV less oxidizing than Y₃₂(O•/OH).^{16, 30} This value is ~ 300–400 mV larger than earlier estimates based on irreversible voltammograms obtained on aqueous NH₂Y and Y.^{4, 17, 37}

The NH₂ conformation of NH₂Y• in RNR

One mechanism of reduction potential perturbation could be associated with protein-induced conformation distortion of RNH₂ ground state and/or RNH₂• state. Our initial HF EPR analysis²⁵ of NH₂ conformation within NH₂Y₇₃₀•-RNR proposed a trigonal pyramidal NH₂Y• ($\theta_1 = 50^\circ$ and $\theta_2 = 30^\circ$) based on early INDO calculations of Loth *et al.*⁵⁹ Later on, DFT calculations by Himo *et al.* predicted a planar structure on the isolated NH₂Y•;⁶⁰ however, a possible role of the protein environment was not discussed.

Subsequently, to examine the detailed electronic structure of the NH₂Y• probe in the protein environment we performed ²H-HF ENDOR spectroscopy of NH₂Y₇₃₀•-α2 and NH₂Y₇₃₁•-α2 combined with quantum chemical calculations.^{26, 27} The calculations predicted an almost planar NH₂Y• structure in all geometry-optimized NH₂Y•-α2 models, which could successfully reproduce the ²H-ENDOR spectra of the exchangeable H bonds. However, a *direct* experimental determination of the ²H-(ND₂) tensor orientations failed because of the line shape distortions associated with the Mims-ENDOR technique and the low S/N of the spectra. Also, a direct measurement of the ¹H-NH hfcs was not feasible because of the background associated with the protons from the surrounding protein environment (4 Å) and from the aromatic ring. Given the new reduction potential, we introduced a new labeling strategy and examined the structure of D₆-NH₂Y• at residue Y₇₃₁, measuring the hfcs of its two amino protons directly. We obtained multi-frequency EPR and 34 GHz ¹H-ENDOR spectra of the D₆-NH₂Y₇₃₁•-RNR that established an almost planar NH₂ conformation ($\theta = 0^\circ \pm 20^\circ$). An alternative method to obtain the angle θ is the use of orientation-selected HF ENDOR spectroscopy, however the low radical yield of D₆-NH₂Y₇₃₁• unfortunately has so far prevented these experiments. We are in the process to introduce CP-edited ENDOR⁷¹ for ²H nuclei, which will potentially facilitate the analysis of ²H-HF ENDOR spectra. Preliminary 94 GHz ²H CP-ENDOR data (not shown) on H₆-ND₂Y₇₃₁•-α2 seem entirely consistent with the present analysis. These experiments should be amenable for analysis of any NH₂Y• in D₂O and do not require a D₆-NH₂Y probe.

While we have not re-analyzed the spectra of NH₂Y₇₃₀•, the quantum chemical calculations supported by simulations of ²H Mims ENDOR spectra²⁶ strongly suggested a nearly planar NH₂Y₇₃₀• conformation. The NH₂Y₃₅₆• in β2 is surrounded by water in a unique environment at the α2β2 interface;³⁴ thus there is still the possibility that NH₂Y₃₅₆• has a different conformation. Unfortunately, incorporation of D₆-NH₂Y₃₅₆ in place of Y₃₅₆ has not been successful so far. From our current EPR analysis we favor the planar conformations for the NH₂Y•s within α2.

We also carried out DFT calculations on 2-aminophenol using the dihedral angles from our initial EPR analysis of NH₂Y₇₃₀• (Scheme S1 and Figures S8 and S9). We used flat and trigonal pyramidal NH₂ conformations and examined different mechanisms, with the ET/PT model of oxidation being the most relevant.⁵⁰ We obtained a range of reduction potential perturbations from conformation to be 190 to 390 mV. Even with the maximum perturbation from the trigonal pyramidal conformation, the reduction potential difference between NH₂Y and Y would still be -197 mV, making NH₂Y-RNR inactive. As a result, the planar NH₂ conformation that we observe ($\theta = 0^\circ \pm 20^\circ$) is also inactive. We also note that the DFT energy minimized structure of the 2-aminophenol radical is planar. The spectroscopic and

DFT analyses together suggest that while the $\text{NH}_2\bullet$ geometric distortion can affect the reduction potential, a planar conformation is present in $\text{NH}_2\text{Y-RNR}$, at least within α .

Activity of $\text{NH}_2\text{Y-RNRs}$?

Given the reduction potential measurement and the NH_2Y planarity in RNR, we assume that each NH_2Y substitution (Figure 2) perturbs the energetics of a given Y in the RT landscape by ~ 587 mV. Our recent studies using many different types of experiments and site-specifically incorporated UAAs (NO_2Y and F_nYs) resulted in the proposal that the energetic landscape between $\text{Y}_{122}\bullet$ in $\beta 2$ and $\text{Y}_{730}\bullet$ in $\alpha 2$ is ~ 200 meV uphill.^{35, 36} We proposed and presented evidence³⁵ that in spite of this barrier, sufficient $\text{C}_{439}\bullet$ in α could be generated to initiate dCDP formation, as rapid and irreversible loss of water during the reduction step drives the reaction to the right. The dCDP formation would occur 10^2 to 10^4 -fold faster than wt-RNR turnover ($\sim 5 \text{ s}^{-1}$) which is conformationally gated.³⁵

If NH_2Y replaces Y_{731} , the ~ 587 mV uphill oxidation of Y_{730} and C_{439} is predicted to give much less $\text{C}_{439}\bullet$ and the predicted rate constant would be 10^2 to 10^4 -fold slower than the measured turnover number of the $\text{NH}_2\text{Y-RNRs}$ ($\sim 0.3 \text{ s}^{-1}$).^{12, 13} With NH_2Y at position Y_{356} , ~ 100 mV more stable than Y_{730} or Y_{731} ,³⁶ the energetic barrier is further increased. From these energetic predictions, we conclude that $\text{NH}_2\text{Y-RNRs}$ should be inactive.

Our previously reported activities for $\text{NH}_2\text{Y-RNRs}$ with CDP and ATP were 3–5% of that reported for wt-RNR.^{12, 13} Given that the purification of $\text{His}_6\text{-NH}_2\text{Y}_{730}\text{-}\alpha 2$ used a Ni^{2+} -affinity column, wt- $\alpha 2$ (untagged) should have been removed.¹³ Thus, the likely source of activity is associated with contaminating wt-RNR that comes from the inability of the evolved tRNA/RS pair to discriminate between NH_2Y and Y at their concentrations inside the cell during the growth conditions. To verify and quantitate Y incorporation, we developed an LC-MS method on the trypsin-digested peptides of $\text{His}_6\text{-NH}_2\text{Y}_{730}\text{-}\alpha 2$. Although this method is not accurate for absolute concentrations, it is robust for the determination of the relative peptide concentrations: P1 to P2.⁷² The 6% of the Y-containing peptide P2 can thus explain the 3–5% steady state turnover with $\text{His}_6\text{-NH}_2\text{Y}_{730(731)}\text{-}\alpha 2$ and $\text{NH}_2\text{Y}_{356}\text{-}\beta 2$.

Our original assessment of $\text{NH}_2\text{Y-RNR}$ activity used two additional methods of analysis. One method takes advantage of the mechanism-based inhibitor 2'-azido-2'-deoxycytidine diphosphate (N_3CDP) which is converted into a nucleotide nitrogen centered radical ($\text{N}\bullet$) in the active site of α ⁷³ and requires chemistry on the nucleotide. The second method, also a single turnover experiment, monitored dCDP formation in the absence of an external reductant in the assay. Theoretically 4 dCDPs/ $\alpha 2$ can be generated, due to the additional two thiols in the C-terminus of each α that re-reduce the active site disulfide and allow formation of a second dCDP (Figure S16).⁷⁴

Results with N_3CDP

With wt-RNR, the reaction with N_3CDP results in 50% $\text{Y}_{122}\bullet$ loss and 50% nucleotide- $\text{N}\bullet$ in the active site, consistent with half-sites reactivity.^{29, 73, 75} The same reaction with $\text{His}_6\text{-NH}_2\text{Y}_{730(731)}\text{-}\alpha 2$, wt- $\beta 2$, ATP, N_3CDP formed $\text{N}\bullet$ at $\sim 15\%$ of the total initial $\text{Y}_{122}\bullet$.¹³ The

6% contaminating wt- $\alpha 2$, assuming this reaction is analogous to the one with the wt RNR, should yield 3% N $\bullet$. Thus, the experimental number is much higher than expected.

Results of one turnover dCDP formation

The wt-RNR single turnover assay, typically gives ~3 dCDPs/ $\alpha 2$ yields instead of the expected 4 dCDPs; diminished yields are attributed to incomplete thiol pre-reduction of $\alpha 2$.⁷⁴ For the His₆-NH₂Y₇₃₀- $\alpha 2$ reaction, the single turnover assays gave ~0.7 dCDPs/ $\alpha 2$. This yield is consistent with ~21% contaminating wt- $\alpha 2$ ^{12, 13}, not the 6% determined above.

Complexity of NH₂Y-RNRs with contaminating wt-RNR

The higher than expected values from the two assays described above were the basis for our original conclusion that NH₂Y-RNRs were active. However, even now knowing that contaminating wt-Y₇₃₀- α is present, we are still unable to explain these observations quantitatively due to the complexity of RNR quaternary structure and stability⁷⁶ and the asymmetry within $\alpha 2\beta 2$.^{29, 73, 75} This complexity is described in the SI as there are too many unknowns to quantitatively assess the previous experimental observations. However, we believe the RNR quaternary structure of the individual subunits and the active complex can rationalize the data. Thus, the mis-incorporation of Y in place of NH₂Y with the suppressor technology using the NH₂Y/NH₂YRS pair, is the key for the low levels of activity. The LC-MS method should be generally useful for measuring mis-incorporation for “large proteins” where ESI-MS on the whole protein does not have the required resolution.

Usefulness of NH₂Y probe in RNR and other proteins

The inactivity of NH₂Y-RNRs is consistent with the thermodynamic landscape we recently proposed for the wt-RNR. Furthermore, RT to NH₂Y inserted at Y₃₅₆, Y₇₃₀ or Y₇₃₁ (Figure 2) still occurs in a kinetically competent fashion in all cases and is the only method to generate sufficient RT pathway radical to study electrostatic environments and hydrogen bonding networks in $\alpha 2$ and to provide insight into the PCET pathway in that subunit.^{26–28} NH₂Y $\bullet$ formation remains a useful method for trapping a tight $\alpha 2\beta 2$ complex²⁴ in a reacted state that resembles a form of RNR in which the RT pathway has been initiated.⁷⁷ Recent studies have also shown that kinetics of NH₂Y $\bullet$ formation in wt and mutant RNRs formed when incubated with of substrate and effector, has revealed important insight about the factors that govern the rate-limiting conformational change(s) that gate RNR turnover.³³

Our studies on NH₂Y-RNRs^{24–28} establish the utility of this probe to study other proteins thought to contain transient Y $\bullet$ s. These systems include photosystem II,⁶² cytochrome oxidase,⁶³ DNA photolyase,⁶⁴ cryptochromes,⁶⁵ prostaglandin synthase,⁶⁶ and BLUF domain proteins.⁶⁷ In addition, Y $\bullet$ s have been implicated in DNA base excision repair enzymes,^{78–80} and in mitigating the damage of off-pathway oxidations in oxidoreductases.^{81–83} The incorporation of NH₂Y in these systems could provide evidence for radical formation in these proteins, and reveal detailed information on electrostatic environments, H-bonding interactions, and kinetics that could provide insight into protein conformational changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Andrew Ullman and Guillaume Passard for helpful discussions. The National Institute of Health (GM079190 (to C.T.), GM47274 (to D.G.N.) and GM29595 (to J.S.)) and the Max Planck Society (DFG-SPP 1601 (to M.B.)) provided funding for this research.

ABBREVIATIONS

$\alpha 2$	RNR large subunit
$\beta 2$	RNR small subunit
$\alpha_3 X$	three helix bundle protein
I^0	electrode surface coverage
CV	cyclic voltammetry
$D_6\text{-NH}_2 Y$	deuterated 3-aminotyrosine
DFT	density functional theory
DPV	differential pulse voltammetry
E	effector
$E^{\bullet\bullet}$	formal reduction potential
ENDOR	electron-nuclear double resonance
E_{pa}	anodic peak potential
E_{pc}	cathodic peak potential
ET	electron transfer
$F_n Y$ (n = 2,3)	fluorotyrosine
GMML	glycerol minimal media leucine
HF EPR	high-field electron paramagnetic resonance
hfc	hyperfine coupling
i_p	peak current
$i_{p, norm}$	normalized peak current
LC-MS	liquid chromatography mass spectrometry
$NH_2 Y$	3-aminotyrosine

NO₂Y	3-nitrotyrosine
PCET	proton-coupled electron transfer
PT	proton transfer
PELDOR	pulsed electron-electron double resonance
PFV	protein film voltammetry
PGE	pyrolytic graphite edge
RNR	ribonucleotide reductase
RS	aminoacyl-tRNA synthetase
RT	radical transport
S	substrate
SWV	square-wave voltammetry
θ	dihedral angle
TCEP	Tris(2-carboxyethyl)phosphine
TR	thioredoxin
TRR	thioredoxin reductase
UAA	unnatural amino acid
W_{1/2}	half-height peak width
wt	wild type
Y•	tyrosyl radical

References

1. Liu CC, Schultz PG. Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* 2010; 79:413–444. [PubMed: 20307192]
2. Yu Y, Cui C, Wang J, Lu Y. Biosynthetic approach to modeling and understanding metalloproteins using unnatural amino acids. *Sci. China Chem.* 2017; 60(2):188–200.
3. Tharp JM, Liu WR. Genetic code expansion: Synthetases pick up the PACE. *Nat. Chem. Biol.* 2017; 13(12):1205. [PubMed: 29161249]
4. Minnihan EC, Nocera DG, Stubbe J. Reversible, Long-Range Radical Transfer in *E. coli* Class Ia Ribonucleotide Reductase. *Acc. Chem. Res.* 2013; 46(11):2524–35. [PubMed: 23730940]
5. Hosseinzadeh P, Lu Y. Design and fine-tuning redox potentials of metalloproteins involved in electron transfer in bioenergetics. *BBA-Bioenergetics.* 2016; 1857(5):557–581. [PubMed: 26301482]
6. Kaila VRI, Verkhovsky MI, Wikström M. Proton-coupled electron transfer in cytochrome oxidase. *Chem. Rev.* 2010; 110(12):7062–7081. [PubMed: 21053971]
7. Blomberg MRA. Mechanism of oxygen reduction in cytochrome c oxidase and the role of the active site tyrosine. *Biochemistry.* 2016; 55(3):489–500. [PubMed: 26690322]

8. Yu Y, Lv X, Li J, Zhou Q, Cui C, Hosseinzadeh P, Mukherjee A, Nilges MJ, Wang J, Lu Y. Defining the role of tyrosine and rational tuning of oxidase activity by genetic incorporation of unnatural tyrosine analogs. *J. Am. Chem. Soc.* 2015; 137(14):4594–4597. [PubMed: 25672571]
9. Oyala PH, Ravichandran KR, Funk MA, Stucky P, Stich TA, Drennan CL, Britt RD, Stubbe J. Biophysical characterization of fluorotyrosine probes site-specifically incorporated into enzymes: *E. coli* ribonucleotide reductase as an example. *J. Am. Chem. Soc.* 2016; 138:7951–7964. [PubMed: 27276098]
10. Minnihan EC, Young DD, Schultz PG, Stubbe J. Incorporation of fluorotyrosines into ribonucleotide reductase using an evolved, polyspecific aminoacyl-tRNA synthetase. *J. Am. Chem. Soc.* 2011; 133(40):15942–15945. [PubMed: 21913683]
11. Yu Y, Zhou Q, Wang L, Liu X, Zhang W, Hu M, Dong J, Li J, Lv X, Ouyang H. Significant improvement of oxidase activity through the genetic incorporation of a redox-active unnatural amino acid. *Chem. Sci.* 2015; 6(7):3881–3885. [PubMed: 26417427]
12. Seyedsayamdost MR, Xie J, Chan CT, Schultz PG, Stubbe J. Site-specific insertion of 3-aminotyrosine into subunit $\alpha 2$ of *E. coli* ribonucleotide reductase: direct evidence for involvement of Y730 and Y731 in radical propagation. *J. Am. Chem. Soc.* 2007; 129(48):15060–71. [PubMed: 17990884]
13. Minnihan EC, Seyedsayamdost MR, Uhlin U, Stubbe J. Kinetics of radical intermediate formation and deoxynucleotide production in 3-aminotyrosine-substituted *Escherichia coli* ribonucleotide reductases. *J. Am. Chem. Soc.* 2011; 133(24):9430–40. [PubMed: 21612216]
14. Seyedsayamdost MR, Reece SY, Nocera DG, Stubbe J. Mono-, di-, tri-, and tetra-substituted fluorotyrosines: new probes for enzymes that use tyrosyl radicals in catalysis. *J. Am. Chem. Soc.* 2006; 128(5):1569–79. [PubMed: 16448128]
15. Ravichandran KR, Liang L, Stubbe J, Tommos C. Formal reduction potential of 3,5-difluorotyrosine in a structured protein: insight into multistep radical transfer. *Biochemistry.* 2013; 52(49)
16. Ravichandran KR, Zong AB, Taguchi AT, Nocera DG, Stubbe J, Tommos C. Formal reduction potentials of difluorotyrosine and trifluorotyrosine protein residues: Defining the thermodynamics of multistep radical transfer. *J. Am. Chem. Soc.* 2017; 139(8):2994–3004. [PubMed: 28171730]
17. DeFelippis MR, Murthy CP, Broitman F, Weinraub D, Faraggi M, Klapper MH. Electrochemical properties of tyrosine phenoxy and tryptophan in indolyl radicals in peptides and amino acid analogs. *J. Phys. Chem.* 1991; 95(8):3416–3419.
18. Stubbe J. Ribonucleotide reductases in the twenty-first century. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95(6):2723–2724. [PubMed: 9501154]
19. Jordan A, Reichard P. Ribonucleotide reductases. *Annu. Rev. Biochem.* 1998; 67:71–98. [PubMed: 9759483]
20. Thelander L. Physicochemical characterization of ribonucleoside diphosphate reductase from *Escherichia coli*. *J. Biol. Chem.* 1973; 248(13):4591–4601. [PubMed: 4578086]
21. Stubbe J, van der Donk WA. Protein radicals in enzyme catalysis. *Chem. Rev.* 1998; 98:705–62. [PubMed: 11848913]
22. Licht S, Stubbe J. Mechanistic investigations of ribonucleotide reductases. *Comp. Nat. Prod. Chem.* 1999; 5:163–203.
23. Uhlin U, Eklund H. Structure of ribonucleotide reductase protein R1. *Nature.* 1994; 370:533–539. [PubMed: 8052308]
24. Minnihan EC, Ando N, Brignole EJ, Olshansky L, Chittuluru J, Asturias FJ, Drennan CL, Nocera DG, Stubbe J. Generation of a stable, aminotyrosyl radical-induced $\alpha 2\beta 2$ complex of *Escherichia coli* class Ia ribonucleotide reductase. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110(10):3835–40. [PubMed: 23431160]
25. Seyedsayamdost MR, Argirevic T, Minnihan EC, Stubbe J, Bennati M. Structural examination of the transient 3-aminotyrosyl radical on the PCET pathway of *E. coli* ribonucleotide reductase by multifrequency EPR spectroscopy. *J. Am. Chem. Soc.* 2009; 131(43):15729–38. [PubMed: 19821570]

26. Argirevic T, Riplinger C, Stubbe J, Neese F, Bennati M. ENDOR spectroscopy and DFT calculations: evidence for the hydrogen-bond network within $\alpha 2$ in the PCET of *E. coli* ribonucleotide reductase. *J. Am. Chem. Soc.* 2012; 134(42):17661–70. [PubMed: 23072506]
27. Nick T, Lee W, Kossmann S, Neese F, Stubbe J, Bennati M. Hydrogen bond network between amino acid radical intermediates on the proton-coupled electron transfer pathway of *E. coli* $\alpha 2$ ribonucleotide reductase. *J. Am. Chem. Soc.* 2015; 137(1):289–98. [PubMed: 25516424]
28. Kasanmascheff M, Lee W, Nick T, Stubbe J, Bennati M. Radical transfer in *E. coli* ribonucleotide reductase: a NH₂Y731/R411- α mutant unmasks a new conformation of the pathway residue 731. *Chem. Sci.* 2016; 7(3):2170–2178. [PubMed: 29899944]
29. Seyedsayamdost MR, Chan CT, Mugnaini V, Stubbe J, Bennati M. PELDOR spectroscopy with DOPA- $\beta 2$ and NH₂Y- $\alpha 2$ s: distance measurements between residues involved in the radical propagation pathway of *E. coli* ribonucleotide reductase. *J. Am. Chem. Soc.* 2007; 129(51):15748–9. [PubMed: 18047343]
30. Berry BW, Martinez-Rivera MC, Tommos C. Reversible voltammograms and a Pourbaix diagram for a protein tyrosine radical. *Proc. Natl. Acad. Sci. U. S. A.* 2012; 109(25):9739–43. [PubMed: 22675121]
31. Ekberg M, Pötsch S, Sandin E, Thunnissen M, Nordlund P, Sahlin M, Sjöberg BM. Preserved catalytic activity in an engineered ribonucleotide reductase R2 protein with a nonphysiological radical transfer pathway - The importance of hydrogen bond connections between the participating residues. *J Biol Chem.* 1998; 273(33):21003–21008. [PubMed: 9694851]
32. Lin Q, Parker MJ, Taguchi AT, Ravichandran KR, Kim A, Kang G, Shao J, Drennan CL, Stubbe J. Glutamate 52- β at the α/β subunit interface of *Escherichia coli* class Ia ribonucleotide reductase is essential for conformational gating of radical transfer. *J. Biol. Chem.* 2017; 292(22):9229–9239. [PubMed: 28377505]
33. Ravichandran KR, Minnihan EC, Lin Q, Yokoyama K, Taguchi AT, Shao J, Nocera DG, Stubbe J. Glutamate 350 plays an essential role in conformational gating of long-range radical transport in *Escherichia coli* class Ia ribonucleotide reductase. *Biochemistry.* 2017; 56(6):856–868. [PubMed: 28103007]
34. Nick TU, Ravichandran KR, Stubbe J, Kasanmascheff M, Bennati M. Spectroscopic Evidence for a H Bond Network at Y356 Located at the Subunit Interface of Active *E. coli* Ribonucleotide Reductase. *Biochemistry.* 2017; 56(28):3647–3656. [PubMed: 28640584]
35. Ravichandran KR, Taguchi AT, Wei Y, Nocera DG, Stubbe J. A >200 meV uphill thermodynamic landscape for radical transport in *E. coli* ribonucleotide reductase determined using fluorotyrosine-substituted enzymes. *J. Am. Chem. Soc.* 2016; 138(41):13706–16. [PubMed: 28068088]
36. Yokoyama K, Smith AA, Corzilius B, Griffin RG, Stubbe J. Equilibration of tyrosyl radicals (Y356•, Y731•, Y730•) in the radical propagation pathway of the *Escherichia coli* class Ia ribonucleotide reductase. *J. Am. Chem. Soc.* 2011; 133(45):18420–32. [PubMed: 21967342]
37. Tommos C, Skalicky JJ, Pilloud DL, Wand AJ, Dutton PL. De novo proteins as models of radical enzymes. *Biochemistry.* 1999; 38(29):9495–507. [PubMed: 10413527]
38. Salowe SP, Stubbe J. Cloning, overproduction, and purification of the B2 subunit of ribonucleoside-diphosphate reductase. *J. Bacteriol.* 1986; 165(2):363–6. [PubMed: 3511029]
39. Yokoyama K, Uhlin U, Stubbe J. A hot oxidant, 3-NO₂Y₁₂₂ radical, unmasks conformational gating in ribonucleotide reductase. *J. Am. Chem. Soc.* 2010; 132(43):15368–79. [PubMed: 20929229]
40. Martinez-Rivera MC, Berry BW, Valentine KG, Westerlund K, Hay S, Tommos C. Electrochemical and structural properties of a protein system designed to generate tyrosine Pourbaix diagrams. *J. Am. Chem. Soc.* 2011; 133(44)
41. Dai QH, Tommos C, Fuentes EJ, Blomberg MRA, Dutton PL, Wand AJ. Structure of a de novo designed model protein of radical systems. *J. Am. Chem. Soc.* 2002; 124:10952–10953. [PubMed: 12224922]
42. Glover SD, Jorge C, Liang L, Valentine KG, Hammarström L, Tommos C. Photochemical tyrosine oxidation in the structurally well-defined $\alpha 3Y$ protein: proton-coupled electron transfer and a long-lived tyrosine radical. *J. Am. Chem. Soc.* 2014; 136(40):14039–14051. [PubMed: 25121576]

43. Saveant JM. Elements of molecular and biomolecular electrochemistry: An electrochemical approach to electron transfer chemistry. John Wiley & Sons, Inc; USA: 2006.
44. Heering HA, Weiner JH, Armstrong FA. Direct detection and measurement of electron relays in a multicentered enzyme: Voltammetry of electrode-surface films of *E. coli* fumarate reductase, an iron– sulfur flavoprotein. *J. Am. Chem. Soc.* 1997; 119(48):11628–11638.
45. Stoll S, Schweiger A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.* 2006; 178(1):42–55. [PubMed: 16188474]
46. Bennati M. EPR Interactions – Hyperfine Couplings. *eMagRes.* 2017; 6:271–282.
47. Mijalis AJ, Thomas DA III, Simon MD, Adamo A, Beaumont R, Jensen KF, Pentelute BL. A fully automated flow-based approach for accelerated peptide synthesis. *Nat. Chem. Biol.* 2017; 13(5): 464–466. [PubMed: 28244989]
48. Wetlaufer DB, Edsall JT, Hollingworth BR. Ultraviolet difference spectra of tyrosine groups in proteins and amino acids. *J. Biol. Chem.* 1958; 233(6):1421–1428. [PubMed: 13610852]
49. Gill SC, Von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 1989; 182(2):319–326. [PubMed: 2610349]
50. Costentin C, Louault C, Robert M, Savéant JM. The electrochemical approach to concerted proton—electron transfers in the oxidation of phenols in water. *Proc. Natl. Acad. Sci.* 2009; 106(43): 18143–18148. [PubMed: 19822746]
51. Tommos C, Valentine KG, Martinez-Rivera MC, Liang L, Moorman VR. Reversible phenol oxidation and reduction in the structurally well-defined 2-mercaptophenol-a3C protein. *Biochemistry.* 2013; 52:1409–1418. [PubMed: 23373469]
52. Glover SD, Tyburski R, Liang L, Tommos C, Hammarström L. Pourbaix Diagram, Proton-Coupled Electron Transfer, and Decay Kinetics of a Protein Tryptophan Radical: Comparing the Redox Properties of W32• and Y32• Generated Inside the Structurally Characterized α3W and α3Y Proteins. *J. Am. Chem. Soc.* 2017; 140(1):185–192. [PubMed: 29190082]
53. Harmalkar SP, Sawyer DT. Electrochemical and spectroscopic studies of 3, 5-di-tert-butyl-2-aminophenol and of electrosynthesized 3, 5-di-tert-butyl-2-iminocyclohexa-3, 5-dienone in aprotic solvents. *J. Org. Chem.* 1984; 49(19):3579–3583.
54. Jackowska K, Bukowska J, Kudelski A. Electro-oxidation of o-aminophenol studied by cyclic voltammetry and surface enhanced Raman scattering (SERS). *J. Electroanal. Chem.* 1993; 350(1–2):177–187.
55. Laviron E. Electrochemical reactions with protonations at equilibrium: Part VII. The 2 e, 1H+ reaction (six-member fence scheme) for a surface or for a heterogeneous reaction in the absence of disproportionation or dimerization. *J. Electroanal. Chem. Interfac. Electrochem.* 1983; 146(1):1–13.
56. Laviron E. Electrochemical reactions with protonations at equilibrium: part VIII. The 2 e, 2H+ reaction (nine-member square scheme) for a surface or for a heterogeneous reaction in the absence of disproportionation and dimerization reactions. *J. Electroanal. Chem. Interfac. Electrochem.* 1983; 146(1):15–36.
57. Mirceski V, Gulaboski R, Lovric M, Bogeski I, Kappl R, Hoth M. Square-wave voltammetry: A review on the recent progress. *Electroanalysis.* 2013; 25(11):2411–2422.
58. Mirceski V, Laborda E, Guziejewski D, Compton RG. New approach to electrode kinetic measurements in square-wave voltammetry: amplitude-based quasireversible maximum. *Anal. Chem.* 2013; 85(11):5586–5594. [PubMed: 23642036]
59. Loth K, Graf F. Structure and Dynamics of Intramolecular Hydrogen Bonds in Radicals: Substituent, steric and solvent effects. *Helv. Chim. Acta.* 1981; 64(6):1910–1929.
60. Himo F, Eriksson LA, Blomberg MRA, Siegbahn PEM. Substituent effects on OH bond strength and hyperfine properties of phenol, as model for modified tyrosyl radicals in proteins. *Int. J. Quant. Chem.* 2000; 76(6):714–723.
61. Bennati M, Murphy DM. Electron paramagnetic resonance: a practitioner's toolkit. Brustolon M, Giamello E, editors John Wiley & Sons; New Jersey: 2009.
62. Barry BA, el-Deeb MK, Sandusky PO, Babcock GT. Tyrosine radicals in photosystem II and related model compounds. Characterization by isotopic labeling and EPR spectroscopy. *J. Biol. Chem.* 1990; 265(33):20139–43. [PubMed: 2173697]

63. Yu MA, Egawa T, Shinzawa-Itoh K, Yoshikawa S, Guallar V, Yeh SR, Rousseau DL, Gerfen GJ. Two tyrosyl radicals stabilize high oxidation states in cytochrome c oxidase for efficient energy conversion and proton translocation. *J. Am. Chem. Soc.* 2012; 134:4753–61. [PubMed: 22296274]
64. Yang L, Nelson RS, Benjdia A, Lin G, Telser J, Stoll S, Schlichting I, Li L. A radical transfer pathway in spore photoproduct lyase. *Biochemistry.* 2013; 52(18):3041–3050. [PubMed: 23607538]
65. Oldemeyer S, Franz S, Wenzel S, Essen L, Mittag M, Kottke T. Essential role of an unusually long-lived tyrosyl radical in the response to red light of the animal-like cryptochrome aCRY. *J. Biol. Chem.* 2016; 291(27):14062–14071. [PubMed: 27189948]
66. Dorlet P, Seibold SA, Babcock GT, Gerfen GJ, Smith WL, Tsai A, Un S. High-field EPR study of tyrosyl radicals in prostaglandin H2 synthase-1. *Biochemistry.* 2002; 41(19):6107–6114. [PubMed: 11994006]
67. Mathes T, Zhu J, van Stokkum IHM, Groot ML, Hegemann P, Kennis JTM. Hydrogen bond switching among flavin and amino acids determines the nature of proton-coupled electron transfer in BLUF photoreceptors. *J. Phys. Chem. Lett.* 2012; 3(2):203–208.
68. Behrens CR, Hooker JM, Obermeyer AC, Romanini DW, Katz EM, Francis MB. Rapid chemoselective bioconjugation through oxidative coupling of anilines and aminophenols. *J. Am. Chem. Soc.* 2011; 133(41):16398–16401. [PubMed: 21919497]
69. Obermeyer AC, Jarman JB, Netirojjanakul C, El Muslemany K, Francis MB. Mild bioconjugation through the oxidative coupling of ortho-aminophenols and anilines with ferricyanide. *Angew. Chem. Int. Ed.* 2014; 53(4):1057–1061.
70. Capehart SL, ElSohly AM, Obermeyer AC, Francis MB. Bioconjugation of gold nanoparticles through the oxidative coupling of ortho-aminophenols and anilines. *Bioconjugate Chem.* 2014; 25(10):1888–1892.
71. Rizzato R, Bennati M. Cross-Polarization Electron-Nuclear Double Resonance Spectroscopy. *ChemPhysChem.* 2015; 16(18):3769–3773. [PubMed: 26503037]
72. Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, Norton S, Kumar P, Anderle M, Becker CH. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal. Chem.* 2003; 75(18):4818–4826. [PubMed: 14674459]
73. Salowe SP, Ator MA, Stubbe J. Products of the inactivation of ribonucleoside diphosphate reductase from *Escherichia coli* with 2'-azido-2'-deoxyuridine 5'-diphosphate. *Biochemistry.* 1987; 26(12):3408–16. [PubMed: 3307907]
74. Ge J, Yu G, Ator MA, Stubbe J. Pre-steady-state and steady-state kinetic analysis of *E. coli* class I ribonucleotide reductase. *Biochemistry.* 2003; 42:10071–83. [PubMed: 12939135]
75. Ravichandran KR, Minnihan EC, Wei Y, Nocera DG, Stubbe J. Reverse electron transfer completes the catalytic cycle in a 2,3,5-trifluorotyrosine-substituted ribonucleotide reductase. *J. Am. Chem. Soc.* 2015; 137(45):14387–95. [PubMed: 26492582]
76. Climent I, Sjöberg BM, Huang CY. Carboxyl-terminal peptides as probes for *Escherichia coli* ribonucleotide reductase subunit interaction: kinetic analysis of inhibition studies. *Biochemistry.* 1991; 30(21):5164–71. [PubMed: 2036382]
77. Wörsdorfer B, Conner DA, Yokoyama K, Livada J, Seyedsayamdost M, Jiang W, Silakov A, Stubbe J, Bollinger JM Jr, Krebs C. Function of the diiron cluster of *Escherichia coli* class Ia ribonucleotide reductase in proton-coupled electron transfer. *J. Am. Chem. Soc.* 2013; 135(23):8585–93. [PubMed: 23676140]
78. Romano CA, Sontz PA, Barton JK. Mutants of the base excision repair glycosylase, endonuclease III: DNA charge transport as a first step in lesion detection. *Biochemistry.* 2011; 50(27):6133–6145. [PubMed: 21651304]
79. Boal AK, Genereux JC, Sontz PA, Gralnick JA, Newman DK, Barton JK. Redox signaling between DNA repair proteins for efficient lesion detection. *Proc. Natl. Acad. Sci.* 2009; 106(36):15237–15242. [PubMed: 19720997]
80. Boal AK, Yavin E, Lukianova OA, O'Shea VL, David SS, Barton JK. DNA-bound redox activity of DNA repair glycosylases containing [4Fe-4S] clusters. *Biochemistry.* 2005; 44(23):8397–8407. [PubMed: 15938629]

81. Gray HB, Winkler JR. Hole hopping through tyrosine/tryptophan chains protects proteins from oxidative damage. *Proc. Natl. Acad. Sci.* 2015; 112(35):10920–10925. [PubMed: 26195784]
82. Zhao X, Yu S, Rangelova K, Suarez J, Metlitsky L, Schelvis JPM, Magliozzo RS. Role of the oxyferrous heme intermediate and distal side adduct radical in the catalase activity of *Mycobacterium tuberculosis* KatG revealed by the W107F mutant. *J. Biol. Chem.* 2009; 284(11): 7030–7037. [PubMed: 19139098]
83. Suarez J, Rangelova K, Jarzecki AA, Manzerova J, Krymov V, Zhao X, Yu S, Metlitsky L, Gerfen GJ, Magliozzo RS. An oxyferrous heme/protein-based radical intermediate is catalytically competent in the catalase reaction of *Mycobacterium tuberculosis* catalase-peroxidase (KatG). *J. Biol. Chem.* 2009; 284(11):7017–7029. [PubMed: 19139099]

Figure 1.

The current mechanistic model for RT in the *E. coli* class Ia RNR^{4, 23} involves the redox active amino acid residues (Y₁₂₂ ↔ [W₄₈?] ↔ Y₃₅₆ in β2 to Y₇₃₁ ↔ Y₇₃₀ ↔ C₄₃₉ in α2). W₄₈, D₂₃₇, E₃₅₀ and E₅₂ are in grey because while their roles in the RT chemistry are important, they remain unknown.^{31–33} Y₃₅₆ in all β2 structures is disordered. The red, blue and purple arrows represent electron transfer, proton transfer and collinear PCET, respectively, of the different PCET mechanisms proposed for individual steps. Two water molecules (grey) are suggested to be involved in PCET at the subunit interface.³⁴

Figure 2.

Proposed thermodynamic landscape for the RT pathway in *E. coli* class Ia RNR based on studies with $NO_2Y_{122}^{\bullet}$ and $F_nY_{122}^{\bullet}$ using the reporter 3,5- F_2Y in place of Y_{356} , Y_{731} and Y_{730} .^{16, 35, 36} Based on measurements on aqueous amino acids,^{4, 17, 37} incorporation of NH_2Y at pathway Y sites was proposed to introduce a thermodynamic sink of -190 to -290 mV.^{12, 13}

Figure 3.

The $2e^-$ redox process of $\alpha_3\text{NH}_2Y$ described as two successive, reversible oxidation-reduction reactions with formal reduction potentials $E^{\circ'}_1$ and $E^{\circ'}_2$. The redox couples for the first and second reactions are denoted here as $\text{NH}Y_{32}(\text{O}/\text{O}\bullet)$ and $\text{NH}_2Y_{32}(\text{O}\bullet/\text{OH})$, respectively.

Figure 4.

Protein film CV on α₃NH₂Y. (A) Raw α₃NH₂Y (red) and α₃F (grey) voltammograms are shown with the corresponding α₃NH₂Y–α₃F difference trace (blue). (B) Background correction done by fitting an asymmetric Gaussian (orange) and a cubic baseline (purple) simultaneously to a raw anodic α₃NH₂Y trace (grey). Data were recorded in 20 mM KP_i, 40 mM KCl, pH 7.1, at 25 °C and using a scan rate of 350 mV/s. (C) Linear CV trumpet plot showing the anodic (E_{pa} , blue) and cathodic (E_{pc} , green) peak potentials vs log of the scan rate. Data were recorded in 20 mM KP_i, 40 mM KCl, pH 6.9, at 25 °C, using a scan rate of 0.25–40 V/s.

Figure 5.

Two successive reversible electron transfers described for the CV of surface-confined redox states. (A) The normalized current (i_{norm}) is depicted as a function of the electrode potential (E) for four different cases of $E^{\circ'}$. The voltammograms are calculated for 25 °C using $E^{\circ'} = E^{\circ'}_1 - E^{\circ'}_2$ of 400 mV (red), 100 mV (blue), 35.6 mV (orange) and -200 mV (black). The middle of each voltammogram corresponds to $(E^{\circ'}_1 + E^{\circ'}_2)/2$. Panel (B) displays the $i_p/i_{p, norm}$ vs $E^{\circ'}$ relationship as a working curve. Panel (C) displays the $W_{1/2}$ vs $E^{\circ'}$ relationship as a working curve. These figures were adapted from ref. 43 and obtained from simulation using DigiElch.

Figure 6.

A) Illustration of $\text{NH}_2\text{Y}^\bullet$ and definition of the θ dihedral angle spanned by the three $\text{C}_4\text{-C}_3\text{-N-H}_{1,2}$ bonds. The $\text{C}_4\text{-C}_3$ and $\text{C}_3\text{-N}$ bonds are in the plane of the phenolic ring (dotted lines). 25 94-GHz EPR spectrum of $\text{D}_6\text{-NH}_2\text{Y}_{731}^\bullet$ in H_2O (black) is shown as compared to simulations (grey). Three simulations, corresponding to pyramidal ($\theta_1 = \theta_2 = 50^\circ$ and $\theta_1 = \theta_2 = 25^\circ$, two upper spectra) and planar ($\theta_1 = \theta_2 = 0^\circ$, lower spectrum) structures of the amino group are displayed. **B)** Illustration of the A tensor direction of $\text{H}^1\text{-NH}$ with respect to the g tensor, with A_x along the NH bond, A_y parallel to the p_z (N) orbital and A_z in the plane.

Figure 7.

34 GHz ^1H -ENDOR spectrum of $\text{D}_6\text{-NH}_2\text{Y}_{731}\bullet$ in H_2O buffer (black) recorded at 100 K and corresponding simulation (grey). The simulation includes hfcs of two amino protons (red and blue) and one perpendicular H-bond (green) according to ref. 27 and the parameters given in Table 1. Additionally, a matrix line was added to reproduce nuclei contributing small hfcs. Experimental conditions: $t_{\pi, \text{prep}} = 100 \text{ ns}$, $t_{\pi, \text{detect}} = 12 \text{ ns}$, $t_{\pi, \text{rf}} = 20 \mu\text{s}$, shot repetition time = 3 ms, shots per point = 1, random rf acquisition, acquisition time/number of scans = 60 h/ 94500 scans.

Figure 8. Standard curve of peptide P1 +2, +3 ions (blue, green) and peptide P2 +2, +3 ions (red, black)

A. The peptides P1 and P2 were prepared in a 1:1 w/w stock solution with predicted extinction coefficients for peptide P1 ($\epsilon_{280\text{ nm}} = 2880\text{ M}^{-1}\text{ cm}^{-1}$) and peptide P2 ($\epsilon_{280\text{ nm}} = 3340\text{ M}^{-1}\text{ cm}^{-1}$). Varying amounts (0.1 ng to 5 ng) of each peptide were analyzed to construct the standard curves. The peak areas were analyzed using the MassHunter workstation software. Peptide P1 preferentially ionized in the +3 charge state (green, 358.52 m/z), rather than the +2 charge state (blue, 537.27 m/z). The peptide P2 ionized preferentially in the +2 state (red, 529.77 m/z), rather than the +3 charge state (black, 353.51 m/z). A linear least squares fit was used for data analysis. The green dashed lines indicate the peak area values corresponding to peptide P1 in the trypsin digested His₆-NH₂Y₇₃₀- α 2 sample. The red dashed lines indicate the peak area values corresponding to peptide P2 in the trypsin digested His₆-NH₂Y₇₃₀- α 2 sample. **B.** The region between 0 ng and 0.5 ng was zoomed in with the same standard curve shown in **A**.

Table 1

Simulated hfcs (MHz) and Euler angles (°) in the D₆-NH₂Y₇₃₁•.

Nuclei	hfcs from multi-frequency ESE simulations			Euler angles (DFT model 3 from ref. 27)			DFT predicted hfcs (model 3 from ref. 27)		
	A _x	A _y	A _z	α	β	γ	A _x	A _y	A _z
¹ H (Amino deuterion 1)	3	-18	-20	-86	98	90	3	-17 (-18)	-22
¹ H (Amino deuterion 2)	5	-18	-24	-96	93	-31	5	-17 (-18)	-26
¹ H (Perpendicular bond)	7	-8	-8.1	-160	110	80	7 (10)	-11 (-10)	-13 (-10)
² H (CB-methylene 1)	1.8	1.5	1.5	-	-	-			
² H (CB-methylene 2)	3.4	3.4	3.4	-	-	-			
¹⁴ N	2.4	1.7	32	60	-5	0			

The uncertainty in the EPR simulated ¹H hfcs is estimated 10 %, uncertainty in the DFT values was reported to 20 %, ²⁷ Best hfcs from ENDOR simulation are in parenthesis with an uncertainty of ≈ 5 %. The uncertainty in the Euler angle α is described in Figure S12.