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## The formal reduction potential of 3,5-difluorotyrosine in a structured protein: Insight into multistep radical transfer

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### Abstract

The reversible Y-O•/Y-OH redox properties of the  $\alpha_3$ Y model protein enable access to the electrochemical and thermodynamic properties of 3,5-difluorotyrosine. The unnatural amino acid has been incorporated at position 32, the dedicated radical site in  $\alpha_3$ Y, by *in vivo* nonsense codon suppression. Incorporation of 3,5-difluorotyrosine gives rise to very minor structural changes in the protein scaffold at pH below the apparent pK ( $8.0 \pm 0.1$ ) of the unnatural residue. Square-wave voltammetry on  $\alpha_3(3,5)\text{F}_2\text{Y}$  provides an  $E^\circ(\text{Y-O}^\bullet/\text{Y-OH})$  of  $1026 \pm 4$  mV *versus* the NHE (pH  $5.70 \pm 0.02$ ) and shows that the fluoro-substitutions lower  $E^\circ$  by  $-30 \pm 3$  mV. These results illustrate the utility of combining the optimized  $\alpha_3$ Y tyrosine radical system with *in vivo* nonsense codon suppression to obtain the formal reduction potential of an unnatural aromatic residue residing within a well-structured protein. It is further observed that the protein  $E^\circ$  values differ significantly from peak potentials derived from irreversible voltammograms of the corresponding aqueous species. This is significant since solution potentials have been the main thermodynamic data available for amino-acid radicals. These findings are discussed relative to recent mechanistic studies on the multistep radical-transfer process in *E. coli* ribonucleotide reductase site-specifically labeled with unnatural tyrosine residues.

Tyrosine serves as a one-electron redox cofactor in catalytic and multistep electron-transfer reactions (1-5). It has been challenging to obtain precise and accurate thermodynamic information for this high-potential protein redox species. Electrochemical characterization of the natural systems has not been feasible due to their size, complexity and sensitivity to oxidative damage. Mechanistic studies on redox proteins employing tyrosine radical (Y•) cofactors must thus partly rely on model systems to provide insights to the thermodynamics involved. Reduction potentials ( $E$  values) of aqueous Y and various analogues have been

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Supporting Information. Materials and Methods;  $\alpha_3(3,5)\text{F}_2\text{Y}$  protein expression analysis (Fig. S1); HPLC and MALDI-TOF evaluation of purified  $\alpha_3(3,5)\text{F}_2\text{Y}$  (Fig. S2); pK<sub>app</sub> fitting analysis (Fig. S3); chemical denaturation plots of  $\alpha_3(3,5)\text{F}_2\text{Y}$  and  $\alpha_3\text{Y}$  at pH 5.0 and 5.5 (Fig. S4);  $\alpha_3(3,5)\text{F}_2\text{Y}$   $I_{\text{net}}$  as a function of  $E_{\text{SW}}$  (Fig S5). This material is available free of charge via the internet at <http://pubs.acs.org>.

obtained by pulse radiolysis and voltammetry methods (6-11). Considerable uncertainty is associated with the reported values. This is in part due to varying experimental conditions, comparison of neutral and zwitterionic amino acids, and complicating issues such as solvent oxidation and the perturbation of solute/working electrode interactions. The most significant uncertainty, however, arises from the reactivity of the radical species themselves. Tyrosine radicals generated in solution will rapidly dimerize ( $\sim 5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ; 12-16) and give rise to quasi/irreversible voltammograms (17, 18). Peak potentials ( $E_{\text{peak}}$ ) derived from such data reflect the electrode process (electrode driven oxidation-reduction of Y) as well as the thermodynamics and kinetics of the coupled side reactions (chemical reduction of Y•). The observed potentials may thus differ by 10s even 100s of millivolts from the formal (true) reduction potential ( $E^{\circ'}$ ) of the Y redox system. Comparison between aqueous solution and protein Y redox chemistry is further complicated by the significant differences in the fundamental properties of these two media (19, 20).

The  $\alpha_3\text{X}$  family of well-structured proteins was developed to address these concerns (10, 21). This model protein system yields thermodynamic information that is uncompromised by electrochemical irreversibility and radical side reactions (22-24). The  $\alpha_3\text{X}$  proteins are based on a common *de novo* designed three-helix bundle scaffold: Scheme 1 displays helical and loop regions in bold and italic, respectively. The N-terminal GS of the 67-residue sequence form part of a thrombin cleavage site and are labeled as -2 and -1 to keep the amino-acid numbering consistent between the chemically synthesized (10) and recombinantly expressed (25, 26)  $\alpha_3\text{X}$  proteins. The buried redox site (position 32 in the middle of the central helix) is occupied by a tryptophan (to form the  $\alpha_3\text{W}$  protein), a tyrosine ( $\alpha_3\text{Y}$ ), or a cysteine ( $\alpha_3\text{C}$ ). C32 has been used to covalently attach phenol (24, 26) and quinone (27) molecules to the protein scaffold. All of the  $\alpha_3\text{X}$  proteins display very similar structural characteristics. They are stable and well-structured from pH ~4 to 10. Their single aromatic residue (W, Y, phenol or quinone) gives rise to UV-Vis, fluorescence and NMR spectra that are highly sensitive to the microenvironment of the redox site. Protein voltammetry has shown that the  $\alpha_3\text{X}$  system displays unique electrochemical properties. The protein scaffold is redox inert to at least +1.3 V vs. NHE (22, 24, 26). The system becomes redox active when a W (10), Y (22, 23), phenol (24, 26) or quinone (27) is introduced at position 32. Fully reversible voltammograms and  $E^{\circ'}$  values have thus far been reported for the Y and phenol-containing  $\alpha_3\text{X}$  proteins (22, 24).

In this study we use the  $\alpha_3\text{X}$  system in combination with the *in vivo* nonsense codon suppression method (28) and square-wave voltammetry (SWV; 17, 29, 30) to determine  $E^{\circ'}$  for a protein 3,5-difluorotyrosine (3,5-F<sub>2</sub>Y) residue. We report  $E^{\circ'}$  between 3,5-F<sub>2</sub>Y and Y residues obtained at highly comparable experimental conditions. The results are compared to published solution potentials and discussed relative to recent mechanistic studies on *E. coli* class Ia ribonucleotide reductase (RNR) site-specifically labeled with unnatural Y residues in an effort to understand the thermodynamic and kinetic landscape of the proton-coupled electron transfer (PCET) pathway in this system (28, 31, 32).

## MATERIALS AND METHODS

### Purification of tyrosine phenol lyase (TPL)

*E. coli* strain SVS370 harboring the plasmid pTZTPL was obtained as a gift from Dr. Robert Phillips (University of Georgia). pTZTPL encodes TPL under a constitutive promoter and the protein was expressed and purified (33, 34) using a slightly modified procedure. The elution fractions from the octyl-sepharose column were assayed using a coupled spectrophotometric assay where a small volume of the fraction was added to an assay mixture containing 2 mM L-tyrosine, 5 mM  $\beta$ -mercaptoethanol, 50  $\mu$ M pyridoxyl-5'-phosphate, 0.3 mg/mL lactate dehydrogenase (*L. leichmannii* from Sigma-Aldrich) and 0.2 mM NADH in 50 mM potassium phosphate pH 8.0. The reaction was monitored at 340 nm for the disappearance of NADH. Fractions containing considerable activity were pooled and concentrated in an Amicon ultrafiltration cell with a YM 10 membrane and the protein was used with no additional purification steps. One unit (U) of activity is defined as 1  $\mu$ mol product/min. A total of 62 U/g cell paste was obtained, in excellent agreement with the previously reported yield (33).

### Enzymatic synthesis of 3,5-difluorotyrosine (3,5-F<sub>2</sub>Y)

TPL was used to enzymatically synthesize 3,5-F<sub>2</sub>Y from 2,6-difluorophenol (the numbering of the aromatic ring changes as the OH carbon is position 1 in the phenol and position 4 in the amino acid). Briefly, a one or two liter reaction mixture containing 10 mM 2,6-difluorophenol, 30 mM ammonium acetate pH 8.0, 60 mM sodium pyruvate, 5 mM  $\beta$ -mercaptoethanol, 40  $\mu$ M pyridoxyl-5'-phosphate and 30 U of TPL at room temperature was stirred in the dark for a total of four days. The mixture was supplied with additional TPL,  $\beta$ -mercaptoethanol and pyridoxyl-5'-phosphate every other day. Once the reaction was complete (as assessed by TLC), the mixture was worked up as described in Ref. 34. The yield is typically > 85% for 3,5-F<sub>2</sub>Y and the final product was characterized by <sup>1</sup>H and <sup>19</sup>F NMR spectroscopy (11, 35).

### Construction of $\alpha_3$ TAG32 by site-directed mutagenesis

The amber stop codon (TAG) was introduced at position 32 of  $\alpha_3$ Y using a modified pET32b- $\alpha_3$ Y plasmid (22, 25) as template and forward primer 5'- GGC GGC CGT ATT GAA GAA CTG AAA AAA AAA TAG GAA GAA CTG AAA AAA AAA ATT GAA GAA CTG GGC GGC GGC-3' and reverse primer 5'- GCC GCC GCC CAG TTC TTC AAT TTT TTT TTT CAG TTC TTC CTA TTT TTT TTT CAG TTC TTC AAT ACG GCC GCC-3'. The mutation was performed using the Stratagene QuikChange kit and confirmed by sequencing at the MIT Biopolymers Laboratory.

### Expression of $\alpha_3(3,5)F_2Y$

pET32b- $\alpha_3$ TAG32 was co-transformed with a plasmid (pEVOL-F<sub>n</sub>Y-RS) encoding the fluorotyrosine tRNA and aminoacyl-tRNA synthetase (F<sub>n</sub>Y-RS) genes (28) into BL21(DE3) competent cells and grown on media containing ampicillin (100  $\mu$ g/mL) and chloramphenicol (35  $\mu$ g/mL) at 37° C. Unnatural amino-acid concentration and protein expression were optimized on a small-scale resulting in the following protocol: 40 mL LB

pre-cultures were started from single colonies, grown for ~ 16 h, and used to inoculate (50-fold dilution) 1 L 2x YT cultures containing 2.0 mM 3,5-F<sub>2</sub>Y. 3,5-F<sub>2</sub>Y additions were made from stock solutions freshly prepared in water, NaOH solubilized and sterile filtered. The expression of F<sub>n</sub>Y-RS was induced at an OD<sub>600</sub> of 0.2-0.3 (L-arabinose, final concentration 0.05% (w/v)). The expression of α<sub>3</sub>(3,5)F<sub>2</sub>Y was induced at an OD<sub>600</sub> of 0.5 (IPTG, final concentration 1mM). Growth was continued for an additional 4 h and the cells were harvested by centrifugation (5000 × g, 15 min, 4° C). Protein expression was monitored by SDS-PAGE. No toxicity was seen due to the unnatural amino acid. A typical yield of 0.5 g cell paste/100 mL and 2.5 g cell paste/L media was obtained for the 0.1 and 1 L cultures, respectively.

### Purification of α<sub>3</sub>(3,5)F<sub>2</sub>Y

Cell paste (~ 5-10 g) was resuspended (5 mL/g paste) in buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9), treated with lysozyme (300 µg/mL, 30 min, 30° C) and lysed by sonication. The lysate was clarified by centrifugation (12000 × g, 20 min, 4° C), passed over a nickel column (10 mL His•bind resin, EMD Millipore) equilibrated with buffer A, and the thioredoxin fusions eluted with a linear 0–40% buffer B (20 mM Tris-HCl, 500 mM NaCl, 1M imidazole, pH 7.9) gradient over 40 min (flow rate 1.5 mL/min). Fractions containing the thioredoxin fusions were identified by SDS-PAGE. Thrombin (T6634; Sigma-Aldrich) was added to the pooled fusion-protein fractions (thrombin/protein ratio 1:2000 (w/w)) and the resulting mixture dialyzed against 20 mM Tris-HCl, 500 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 8.0 at RT for >16 h. The thrombin digestion produces three major products: His- tagged thioredoxin, the truncated α<sub>3</sub>(residue 1–31) peptide (MW 3579 Da) and the full-length α<sub>3</sub>(3,5)F<sub>2</sub>Y protein (MW 7557 Da). A major fraction of the truncated α<sub>3</sub>(residue 1–31) product was removed during the overnight dialysis step (Spectra/Por tubing MWCO 3500 Da). The digestion/dialysis mixture (~ 30 mL) was passed over a nickel column (10 mL His•bind resin equilibrated with buffer A) to remove the His-tagged thioredoxin and any remaining undigested fusion products. α<sub>3</sub>(3,5)F<sub>2</sub>Y (sample injection volume 5-10 mL) was isolated by reversed-phase HPLC (218TP C18 column, particle size 10 µm, column size 10 × 250 mm; Grace/VYDAC) using a linear water/acetonitrile/0.1% (w/v) trifluoroacetic acid gradient (30-60% acetonitrile over 45 min, flow rate 5 mL/min), and stored as lyophilized powder. The protein purification steps were monitored by SDS-PAGE. Purity was evaluated by reversed-phase HPLC (218TP C18 column, particle size 5 µm, column size 4.6 × 250 mm; Grace/VYDAC) using a linear water/acetonitrile/0.1% (w/v) trifluoroacetic acid gradient (20-70% acetonitrile over 50 min, flow rate 1 mL/min). Molecular weights were verified by matrix-assisted desorption/ionization-time of flight (MALDI-TOF) mass spectrometry using a Bruker Microflex 3.1. An average yield of ~3 mg pure α<sub>3</sub>(3,5)F<sub>2</sub>Y/L culture was observed.

### Absorption spectroscopy

Absorption spectra were recorded on a Varian Cary 50 Bio UV-Vis spectrometer at RT. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) with standard curves prepared using either bovine serum albumin (for thioredoxin fusion samples) or α<sub>3</sub>W (10) (for α<sub>3</sub>Y and α<sub>3</sub>(3,5)F<sub>2</sub>Y protein-characterization samples). An ε<sub>280</sub> of 5690 M<sup>-1</sup> cm<sup>-1</sup> was used for α<sub>3</sub>W (10, 36, 37) when preparing the standard curves. The

apparent tyrosinate/tyrosine  $pK_a$  ( $pK_{app}$ ) of (3,5)F<sub>2</sub>Y<sub>32</sub> was measured by dissolving lyophilized  $\alpha_3(3,5)F_2Y$  in 3.0 mL 20 mM sodium acetate, 20 mM potassium phosphate, 20 mM sodium borate (APB buffer), pH 12 to give an absorption of 0.2 at 277 nm (10 mm path). The protein stock solution was added to 20 mM APB buffer generating two samples with pH ~ 5 and 12, respectively. The pH was pre-adjusted in the APB buffer samples to generate the final pH upon addition of the protein stock. The protein dilution was 2-fold providing a final 277 nm absorption of 0.1 (10 mm path; pH 12). Equal-volume (1.6 mL) titration was performed manually by removing a 100-400  $\mu$ L portion from the cuvette containing the high-pH sample and then adding the same amount of low-pH sample per pH adjustment. The  $pK_{app}$  was obtained by fitting the pH-induced increase in the (3,5)F<sub>2</sub>Y<sub>32</sub>-O<sup>-</sup> absorption to a single  $pK_a$ .

### Circular dichroism (CD) spectroscopy

CD data were collected on an Aviv 202 CD spectrometer at 25° C. The instrument was equipped with an automated titration system. For the  $\alpha$ -helical measurements, CD spectra were collected from  $\alpha_3W$ ,  $\alpha_3Y$  and  $\alpha_3(3,5)F_2Y$  dissolved in 20 mM sodium acetate, pH 5.6. Protein concentrations were determined by the Bradford assay and using  $\alpha_3W$  as the standard. pH titrations were performed by dissolving lyophilized protein in 20 mM APB buffer, pH 4.8 to a 235 nm ellipticity of ~-175 mdegrees (10 mm path). The protein stock solution was added to 20 mM APB buffer generating two samples with pH ~ 4 and 10, respectively. The pH was pre-adjusted in the APB buffer samples to generate the final pH upon addition of the protein stock. The protein dilution was 4-fold generating a final 222 nm ellipticity ~-150 mdegrees (10 mm path; pH 4). Equal-volume (2.5 mL) titration was performed manually by removing a 40-1000  $\mu$ L portion from the cuvette containing the high-pH sample and then adding the same amount of low-pH sample per pH adjustment. Protein stability measurements were conducted by dissolving lyophilized protein in 20 mM sodium acetate, 20 mM potassium phosphate (AP buffer), pH 5.0 or 5.5 buffer to a 230 nm ellipticity of -75 to -140 mdegrees (1 mm path). The protein stock solution was added to 20 mM AP buffer containing 0 and 9.5 M urea, respectively. The pH was 5.0 or 5.5 in the protein/AP buffer and protein/AP buffer/urea solutions. The protein dilution was 18-fold generating a final 222 nm ellipticity in the -110 to -210 mdegrees range (10 mm path) at zero molar denaturant. The urea denaturation experiments were performed by automated equal-volume (2.0 mL) titration controlled from the Aviv software. Global stability values were determined by fitting the denaturation curves as described in Ref. 38.

### Square-wave voltammetry (SWV)

Voltammetry measurements (17, 29, 30) were performed using an Autolab PGSTAT12 potentiostat equipped with a temperature-controlled, Faraday-cage protected three-electrode micro-cell (Princeton Applied Research). The Ag/AgCl reference electrode and the platinum wire counter electrode (Advanced Measurements Inc.) were stored dry and prepared by filling the former with a 3M KCl/saturated AgCl solution and the latter with sample buffer. All measurements were carried out using a 3 mm diameter pyrolytic graphite edge (PGE) working electrode (Bio-Logic, USA). The electrode surface was activated between measurements by manually polishing its surface for 60 s in a 1.0  $\mu$ m diamond/water slurry on a diamond polishing pad (Bio-Logic, USA) followed by 60 s in a 0.05  $\mu$ m alumina/water

slurry on a microcloth pad (Bioanalytical systems Inc.). The electrode was rinsed with an excess of methanol followed by milli-Q water directed against the surface of the electrode. Measurements were performed immediately following the polishing procedures. Solution resistance was compensated for by using the positive feed-back iR compensation function of the Autolab system. Potentials are given *versus* the normal hydrogen electrode (NHE). All samples were prepared from ultra-pure chemicals and the measurements performed under an argon atmosphere. Preparation of the voltammetry protein samples is described in the main text. Protein concentration series were obtained by stepwise dilution of the protein samples in the electrochemical cell while keeping the sample volume constant at 2.5 mL. The pH was monitored directly in the electrochemical cell using a pH microelectrode (Microelectrodes Inc.) connected to a SevenMulti pH meter (Mettler Toledo). The pH electrode was disconnected from the pH meter during the active measurements to avoid the risk of introducing electric noise. Data processing and analyses were performed using the Autolab GPES software and PeakFit (Systat Software Inc.).

## RESULTS

### $\alpha_3(3,5)F_2Y$ expression and purification

3,5-difluorotyrosine was synthesized enzymatically (33, 34) as described in the Materials and Methods section. The amber stop codon (TAG) was introduced in place of the codon for residue 32 (Scheme 1) in a modified pET32b plasmid used for expression of the  $\alpha_3X$  proteins (22, 25). The  $\alpha_3TAG$  variant was co-expressed with an evolved polyspecific fluorotyrosine aminoacyl-tRNA synthetase (28) in *E. coli* BL21(DE3) cells. Protein expression was initially evaluated in 100 mL growth cultures by SDS-PAGE and mass spectrometry. Optimal expression was observed with 2.0 mM 3,5- $F_2Y$  in 2x YT media (see Fig. S1 in the Supporting Information). The growth cultures were scaled to 1 L to generate sufficient material for protein characterization and voltammetry studies. Protein expression and purification protocols are described in detail in Materials and Methods. The purity of the  $\alpha_3(3,5)F_2Y$  preparations was evaluated by reversed-phase HPLC (Fig. S2A) and the correct protein molecular weight was verified by mass spectrometry (Fig. S2B). The average yield of pure  $\alpha_3(3,5)F_2Y$  was ~3 mg/L culture, which represents a suppression efficiency of only ~10%. Efforts to improve the suppression efficiency are in progress.

### Protein characterization

The aims of this study are to determine (i) the electrochemical reversibility of  $\alpha_3(3,5)F_2Y$ , (ii)  $E^{o'}$  of (3,5) $F_2Y_{32}$ , and (iii)  $E^{o'}$  between (3,5) $F_2Y_{32}$  and  $Y_{32}$  when recorded at near identical conditions. Initially we compared the structural properties of  $\alpha_3Y$  and  $\alpha_3(3,5)F_2Y$ .  $\alpha_3Y$  is highly helical and well-structured from pH 4.5 to 10 (10, 22). The apparent  $Y-O^-/Y-OH$   $pK_a$  ( $pK_{app}$ ) of  $Y_{32}$  is  $11.3 \pm 0.1$  (Table 1). Exchanging  $Y_{32}$  to (3,5) $F_2Y_{32}$  was not expected to significantly perturb the protein scaffold (39, 40) at pH conditions where the buried phenol side chain is uncharged. Experiments were carried out to determine a suitable pH range for the SWV analysis, i.e., where (3,5) $F_2Y_{32}$  remains protonated and the structural properties of  $\alpha_3(3,5)F_2Y$  closely resemble those of  $\alpha_3Y$ .



Figure 1A displays absorption spectra of  $\alpha_3(3,5)F_2Y$  collected over a pH 5.7 (grey) to 10.4 (purple) range. The  $(3,5)F_2Y_{32-O^-}/(3,5)F_2Y_{32-OH}$  transition titrates with a  $pK_{app}$  of  $8.0 \pm 0.1$  (see Fig. S3 for details). The protein environment increases the  $pK_{app}$  by  $\sim 1$  unit relative to aqueous 3,5- $F_2Y$  ( $pK_a$   $7.0 \pm 0.2$ ; Table 1; 11, 35). The weak and blue shifted absorbance of aqueous 3,5- $F_2Y-OH$  ( $\epsilon_{263}$   $560 M^{-1} cm^{-1}$ ) relative to unmodified  $Y-OH$  ( $\epsilon_{275}$   $1490 M^{-1} cm^{-1}$ ; Table 1) was expected to translate into a rather poorly defined absorption spectrum of  $\alpha_3(3,5)F_2Y$ . The blue shift increases the spectral overlap between the absorption of the aromatic side chain and the large absorption of the protein backbone ( $\lambda_{max}$  190-220 nm) making  $\alpha_3(3,5)F_2Y$  concentration measurements based on UV absorbance unreliable. Thus, the Bradford method was used to determine protein concentrations with standard curves prepared from  $\alpha_3W$ .  $\epsilon_{277}$  of  $1500 \pm 80$  (pH 5.4) and  $\epsilon_{294}$  of  $2430 \pm 130$  (pH 13.4)  $M^{-1} cm^{-1}$  were obtained for  $Y_{32-OH}$  and  $Y_{32-O^-}$  in  $\alpha_3Y$ , respectively. The  $\epsilon_{277}$  value is consistent with the average  $\epsilon_{280}$  of  $1490 M^{-1} cm^{-1}$  typically used for protein  $Y-OH$  residues (36, 37) and close to the  $\epsilon_{max}$  values of aqueous  $Y-OH$  (Table 1; 11). We conclude that the Bradford assay determines the  $\alpha_3X$  protein concentration with good accuracy.

CD spectroscopy was used to measure the absolute  $\alpha$ -helical content, the pH sensitivity of this parameter, and the global stability of  $\alpha_3(3,5)F_2Y$  relative to  $\alpha_3Y$  (Table 1). Figure 1B displays far-UV CD spectra of  $\alpha_3(3,5)F_2Y$  (blue) and  $\alpha_3Y$  (red) collected at pH 5.6. The reference spectrum of the structurally characterized  $\alpha_3W$  protein (green; 10, 25) is also shown. The protein concentration in the CD samples was determined by the Bradford method. The CD spectral line shapes are essentially identical and display the characteristic 208/222 nm double minima of a predominantly  $\alpha$ -helical protein. The 222 nm mean residue molar ellipticity ( $[\Theta]_{222}$ ) reflects the  $\alpha$ -helical content, which was estimated to  $\sim 75$ -80% for  $\alpha_3(3,5)F_2Y$  and  $\alpha_3Y$  at pH 5.6 (Table 1). This translates into  $50 \pm 1$  residues with helical  $\psi$  and  $\phi$  backbone angles in  $\alpha_3(3,5)F_2Y$  and  $\alpha_3Y$ . This degree of helicity has consistently been observed for structurally characterized  $\alpha_3X$  proteins ( $\alpha_3W$  PDB ID 1LQ7 (25); 2-mercaptophenol- $\alpha_3C$  2LXY (24)). Figure 1C displays  $[\Theta]_{222}$  and the corresponding % helix of  $\alpha_3(3,5)F_2Y$  as a function of pH. The  $\alpha$ -helical content of  $\alpha_3(3,5)F_2Y$  decreases by only 2% from pH 4.7 to 6.7 and by 6% from pH 6.7 to 10. These small pH-induced changes in helical content are likely to be driven by the deprotonation of  $(3,5)F_2Y_{32}$ . The global stability of  $\alpha_3(3,5)F_2Y$  and  $\alpha_3Y$  was thus compared at acidic pH  $>$  two pH units below the  $pK_{app}$  of  $(3,5)F_2Y_{32}$  (Fig. S4). The stability of the two proteins was found to be identical at pH 5.0 and differ by only  $0.2 kcal mol^{-1}$  at pH 5.5 (Table 1). The SWV analysis described below was conducted at low pH ( $5.6 \pm 0.1$ ) where the structural properties of  $\alpha_3(3,5)F_2Y$  and  $\alpha_3Y$  are very similar and their phenols are protonated.

### Preparation of SWV samples

The protein concentration is a key parameter to control when investigating the protein/working electrode interactions and optimizing the Faradaic response (23, 24). The high  $\alpha$ -helical content of  $\alpha_3(3,5)F_2Y$  makes CD spectroscopy a precise method to determine the protein concentration in a highly reproducible manner. Voltammetry samples were thus prepared by dissolving lyophilized protein in buffer until an appropriate  $\theta_{222}$  and the protein concentration was calculated from the determined  $[\Theta]_{222}$  value (see Fig. 1 legend and Table

1). The supporting electrolyte, KCl, was added to the samples after the CD measurements to avoid UV absorption of the chloride ion.

### SWV analysis of $\alpha_3(3,5)F_2Y$

SWV voltammetry was conducted using a PGE working electrode. In SWV, the base potential ( $E_{\text{step}}$ ) is changed incrementally in a series of forward and reverse pulses (17, 29, 30). The pulse height and length are set by the SW amplitude ( $E_{\text{SW}}$ ) and frequency ( $f$ ), respectively. The current is sampled at the end of each alternating pulse and traced out as a function of  $E_{\text{step}}$ . The pulse train generates a forward ( $I_{\text{for}}$ ), a reverse ( $I_{\text{rev}}$ ), and a net ( $I_{\text{net}} = I_{\text{for}} - I_{\text{rev}}$ ) voltammogram. Previous studies using the PGE electrode have shown that ~20–100  $\mu\text{M}$   $\alpha_3X$  in 20 mM APB buffer and 60–140 mM KCl yields Y and phenol voltammograms with optimal S/N at both acidic (5.5) and alkaline (8.5) pH (23, 24). At these conditions, the system is governed by diffusion-controlled kinetics, the protein does not unfold on the electrode surface, and the observed potential is independent of functional groups present at the electrode surface. The  $\alpha_3(3,5)F_2Y$  SWV measurements were consequently conducted using 20 mM APB and 75 mM KCl. Overall,  $\alpha_3(3,5)F_2Y$  displays electrochemical properties very similar to those of  $\alpha_3Y$  (23). The Faradaic response is consistent with diffusion-controlled kinetics (Fig. S5) and benign  $\alpha_3(3,5)F_2Y$ /working electrode interactions (Figs. 2A and B). Figure 2C shows the change in the peak potential of the forward ( $I_{\text{for}}$ ), reverse ( $I_{\text{rev}}$ ) and net ( $I_{\text{net}}$ ) currents as a function of the SW frequency. Background-corrected voltammograms from this data series are shown in Figs. 2D-F.  $E_{\text{for}}$ ,  $E_{\text{rev}}$  and  $E_{\text{net}}$  level off and come together as the SW frequency increases. This is consistent with a redox system that is shifting from the upper quasi-reversible region towards the fully reversible region as the SW frequency increases (29, 30).  $E_{\text{net}}$  of  $1026 \pm 3$  mV,  $E_{\text{for}}$  of  $1021 \pm 3$  mV, and  $E_{\text{rev}}$  of  $1032 \pm 2$  mV are observed for the 270–540 Hz range at  $\text{pH } 5.70 \pm 0.02$ . The observed frequency insensitivity and small separation in peak maxima mean that  $E_{\text{net}}$  closely approximates  $E^{\circ'}$  (i.e. within a few mV; 23, 24, 30). With a  $\text{p}K_{\text{app}}$  of 8.0, we calculate  $E^{\circ'}$ (pH 7.0) to be 952 mV (Table 1). Comparing these results with the earlier reported  $\alpha_3Y$  SWV study (23), provides an  $E^{\circ'}$  between  $Y_{32}$  and  $(3,5)F_2Y_{32}$  of  $-28$ – $33$  mV. This result was reproduced in a control experiment where  $\alpha_3(3,5)F_2Y$  and  $\alpha_3Y$  SW voltammograms were collected at identical experimental conditions, using the same electrode setup (Fig. 3,  $E_{\text{net}} -30 \pm 3$  mV). We conclude that  $E^{\circ'}$  of the  $Y_{32}\text{-O}\bullet/Y_{32}\text{-OH}$  redox pair is lowered by  $30 \pm 3$  mV upon fluoro-substitution at ring positions 3 and 5 (carbon atoms next to the phenol oxygen). We further note that the difference between  $E_{\text{peak}}$  of aqueous Y and  $E^{\circ'}$  of  $\alpha_3Y$  is  $-150$  mV, with the protein being the more oxidizing system (Table 1).

## DISCUSSION

### Measuring tyrosine reduction potentials

There are three basic properties associated with tyrosine that make electrochemical characterization a major challenge. The measurements must be carried out at highly oxidizing conditions ( $\sim 1.0 \pm 0.3$  V vs. NHE), the  $Y\bullet$  state is reactive (e.g. 12–16), and both the reduced ( $\epsilon_{280}$   $1490 \text{ M}^{-1} \text{ cm}^{-1}$ ; 36, 37) and oxidized ( $\epsilon_{408}$  of  $2750 \text{ M}^{-1} \text{ cm}^{-1}$ ; 41) states have weak extinction coefficients. The combination of these three properties rule out redox



titration as a viable method to study the thermodynamics of Y redox cofactors. This is significant since redox titration, using either chemical titrants and a redox cuvette or a potentiostat-controlled spectroelectrochemical cell, is the most common approach to measure reduction potentials of protein redox cofactors. The two former properties also make voltammetry a challenge. Measurements performed in the + 1.0 V range will generate background currents arising from the working electrode and from the water solvent itself. These background currents will compromise data analysis unless the Faradaic current reflecting the Y redox cofactor is prominent. Moreover, uncontrolled oxidation of surface residues and general oxidative damage to the host protein are likely events. In addition to these concerns are the inherent issues associated with protein voltammetry, i.e., to identify conditions for which the folded protein exhibits direct and reversible electron transfer between the working electrode and the redox site of interest (42-44). These are the main reasons that a direct voltammetry approach on a complex radical enzyme such as *E. coli* RNR is not practical or even possible.

The  $\alpha_3X$  system was developed to address these experimental barriers and allow rigorous electrochemical characterization of aromatic amino acids buried within a structured protein. In three recent studies (22-24), we have demonstrated that pulsed voltammetry methods (differential pulse voltammetry and SWV) generate reversible Y and phenol protein voltammograms of high quality. Control studies have shown that the characteristics of the  $\alpha_3X$  voltammograms are highly reproducible and that they uniquely reflect the aromatic residue at position 32 and its local environment. The more commonly used method of cyclic voltammetry (17) generates a poor Faradaic response from the  $\alpha_3X$  proteins (22) and this method was deemed too insensitive for this system. We note that the potential range probed in this study is very oxidizing for a protein system with measured  $E_{\text{net}}$  values well above +1.0 V. The high quality of the presented voltammograms is the result of combining the sensitivity provided by the pulsed SWV method (due to effective elimination of capacitive background currents) with carefully optimized protein/PGE working electrode conditions. The latter include electrode polishing procedures (described in the Materials and Methods sections), the sample composition (optimized for the  $\alpha_3X$ /PGE system in Refs. 23 and 24), and the protein concentration (Fig. 2A). Importantly, the electrochemical reversibility observed for the  $\alpha_3X$  proteins reflects the long halftimes (> 100s of ms) of the radicals generated in this system (23, 24). Thus, the protein environment efficiently blocks the deleterious Y• side reactions that typically compromise electrochemical characterization of the solvated species.

In this study the optimized  $\alpha_3X$  radical system was combined with *in vivo* nonsense codon suppression (28) to measure  $E^{\circ}$  of a protein 3,5-F<sub>2</sub>Y residue. The use of unnatural amino acids to study protein electron-transfer (ET) and proton coupled electron-transfer (PCET) processes involving Y redox sites has emerged as an informative experimental approach (31, 45, 46). The Y analogues provide a means to introduce major changes in the  $pK_a$  of the reduced state and in the  $E^{\circ}$  values of the Y-O•/Y-OH and Y-O•/Y-O<sup>-</sup> redox couples. The  $pK_a$  of the oxidized state (-2 for aqueous Y-OH•+; 47) is predicted to be non-accessible within the structural and catalytic pH ranges of proteins. Residue 32 resides in a structured, solvent-protected and low dielectric site typical of natural Y redox cofactors. The  $\alpha_3X$

radical system will be used to generate a consistent set of  $E^{\circ'}$  values to provide a guide for interpreting mutation-induced changes in the free-energy profile of native ET/PCET chains involving aromatic amino-acid redox site(s). The availability of protein  $E^{\circ'}$  values removes the uncertainty associated with using solution potentials to interpret protein redox events. Table 1 shows that there are considerable differences in protein potentials obtained under reversible conditions relative to solution potentials obtained under irreversible conditions. We find a difference between  $E^{\circ'}$  (protein Y<sub>32</sub> and 3,5-F<sub>2</sub>Y<sub>32</sub>,  $-30 \pm 3$  mV) and  $E_{\text{peak}}$  (aqueous Y and 3,5-F<sub>2</sub>Y,  $-60$  mV) of 30 mV (Table 1). This difference most certainly arises from the irreversible characteristics of the small-molecule measurements. We also found that the absolute values of  $E^{\circ'}$  (protein Y<sub>32</sub> and 3,5-F<sub>2</sub>Y<sub>32</sub>) vs.  $E_{\text{peak}}$  (aqueous Y and 3,5-F<sub>2</sub>Y) is rather large ( $-150$ - $180$  mV). This observation suggests that the influence by the protein matrix on the absolute potentials of radical cofactors may be substantial. We conclude that site-specific incorporation of an unnatural amino acid into the electrochemically reversible  $\alpha_3X$  redox system is a feasible and informative approach. It will be expanded to include other Y analogues in future studies, as described in more detail below.

### Using the $\alpha_3X$ system as a guide to interpret mechanistic studies of E. coli RNR

RNRs catalyze the formation of deoxynucleotides (dNDPs) from their corresponding nucleotides (1, 31). *E. coli* class Ia RNR is composed of two homodimeric subunits,  $\alpha_2$  and  $\beta_2$ . A stable diferric-Y<sub>122</sub><sup>•</sup> cofactor in  $\beta_2$  generates a transient cysteine radical (C<sub>439</sub><sup>•</sup>) in the active site of  $\alpha_2$  located 35 Å away. The reversible long range radical-transfer process, triggered by substrate and effector binding to  $\alpha_2$ , is proposed to involve multiple PCET steps via a conserved pathway (Y<sub>122</sub>  $\rightleftharpoons$  [W<sub>48</sub>]  $\rightleftharpoons$  Y<sub>356</sub> in  $\beta_2$  to Y<sub>731</sub>  $\rightleftharpoons$  Y<sub>730</sub>  $\rightleftharpoons$  C<sub>439</sub> in  $\alpha_2$ ; 31). We have recently described the site-specific insertion of 3-nitrotyrosine (NO<sub>2</sub>Y) in place of Y<sub>122</sub> in  $\beta_2$  (48). The diferric-NO<sub>2</sub>Y<sub>122</sub><sup>•</sup> cofactor was generated and studies with  $\alpha_2$ , substrate (CDP) and effector (ATP) allowed the first observation of a transient, kinetically competent Y<sup>•</sup> on pathway by electron paramagnetic resonance (EPR) spectroscopy (48). Pulsed electron-electron double resonance (PELDOR) spectroscopy was used to establish the primary location of the new radical as Y<sub>356</sub><sup>•</sup>- $\beta_2$  and suggested the formation of a small percentage of radical at either Y<sub>731</sub> or Y<sub>730</sub> in  $\alpha_2$ . To probe this further, the reaction of diferric-NO<sub>2</sub>Y<sub>122</sub><sup>•</sup>- $\beta_2$  with (3,5)F<sub>2</sub>Y<sub>731</sub>- $\alpha_2$  (or (3,5)F<sub>2</sub>Y<sub>730</sub>- $\alpha_2$ ), CDP and ATP was performed. Analysis of the reactions by X-band EPR spectroscopy demonstrated an equilibrium between Y<sub>356</sub><sup>•</sup> in  $\beta_2$  and (3,5)F<sub>2</sub>Y<sub>731</sub><sup>•</sup> or (3,5)F<sub>2</sub>Y<sub>730</sub><sup>•</sup> in  $\alpha_2$  with 85-90% of the spin localized at Y<sub>356</sub> and 15-10% distributed over F<sub>2</sub>Y<sub>731</sub>/F<sub>2</sub>Y<sub>730</sub> (32). To extrapolate the equilibrium observed with 3,5-F<sub>2</sub>Y to Y, the native pathway residue, requires a knowledge of  $E^{\circ'}$  values for 3,5-F<sub>2</sub>Y and Y. To date only solution  $E_{\text{peak}}$  values derived from irreversible voltammograms have been available for the fluorotyrosines (11, 31). The SWV measurements performed on  $\alpha_3Y$  and  $\alpha_3(3,5)F_2Y$  provide the first  $E^{\circ'}$  values representing the radical species in a well-defined protein environment. The small  $E^{\circ'}$  of  $-30 \pm 3$  mV between Y<sub>32</sub> and 3,5-F<sub>2</sub>Y<sub>32</sub> suggests that a thermodynamic landscape, formed by three transient tyrosyl radicals (Y<sub>356</sub>, Y<sub>731</sub>, Y<sub>730</sub>) of similar energy with one (Y<sub>356</sub>) being most prevalent, is a reasonable depiction of nature's design within this part of the pathway.

## Future perspectives

We have shown that formal reduction potentials representing a reversible Y-O•/Y-OH redox system can be obtained for  $\alpha_3Y$  (23) and  $\alpha_3(3,5)F_2Y$  (this work). Future studies will involve the incorporation of other modified amino acids at position 32 in the  $\alpha_3X$  scaffold to obtain a consistent set of  $E^{\circ'}$  values from Y and modified Y residues. To determine  $E^{\circ'}$  of 3-aminotyrosine (NH<sub>2</sub>Y) and 2,3,5-trifluorotyrosine (2,3,5F<sub>3</sub>Y) incorporated into the  $\alpha_3X$  protein is of particular interest. In the former case, studies with NH<sub>2</sub>Y in place of Y<sub>356</sub>, Y<sub>731</sub> and Y<sub>730</sub> show an active pathway and that dNDPs can be produced (49). This is an interesting observation since the solution potential of NH<sub>2</sub>Y (9) suggests that this species may act as a radical sink shutting down the pathway, as observed in experiments on 3,4-dihydroxyphenylalanine (DOPA)-labeled Y<sub>356</sub>- $\beta 2$  (50). Obtaining a solid protein  $E^{\circ'}$  value for NH<sub>2</sub>Y<sub>32</sub> vs. Y<sub>32</sub> would be valuable for interpreting the characteristics of the NH<sub>2</sub>Y-labeled RNR systems (see Ref. 31 for more details). In the latter case, experiments are in progress in an effort to measure the thermodynamics between Y<sub>122</sub> and Y<sub>356</sub> in  $\beta 2$ . Toward that goal, diferric-F<sub>n</sub>Y<sub>122</sub>• (n = 2 or 3)- $\beta 2$ s have been successfully generated and the reactions of (3,5)F<sub>2</sub>Y<sub>122</sub>•- $\beta 2$  and (2,3,5)F<sub>3</sub>Y<sub>122</sub>•- $\beta 2$  with  $\alpha 2$ , CDP and ATP have been analyzed by EPR spectroscopy (28, 31). These studies reveal that only (2,3,5)F<sub>3</sub>Y<sub>122</sub>• is capable of generating a new pathway radical that is postulated to be Y<sub>356</sub>•. Detailed investigations are ongoing with F<sub>n</sub>Y- $\beta 2$ s and wt- $\alpha 2$  and Y<sub>731</sub>F- $\alpha 2$  to establish if there is an equilibrium between Y<sub>356</sub>• and F<sub>n</sub>Y<sub>122</sub>. Thus, protein  $E^{\circ'}$  values of (2,3,5)F<sub>3</sub>Y and other F<sub>n</sub>Y residues relative to Y may also play an important role in defining the thermodynamic landscape within this part of the pathway. Finally, work to complement the  $\alpha_3X$  protein  $E^{\circ'}$  data series with  $\alpha_3W$  is also in progress. Tryptophan is an important protein redox cofactor observed in a number of enzymes (e.g. 51-53), proposed to participate in the *E. coli* RNR radical-transfer pathway (54, 55), and engineered to study multistep electron tunneling in *Pseudomonas aeruginosa* azurin (56).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

$\alpha 2$ and $\beta 2$	subunits of <i>E. coli</i> RNR
$\alpha_3Y$	$\alpha_3W$ and $\alpha_3C$ , <i>de novo</i> protein containing a single buried tyrosine, tryptophan or cysteine
$\alpha_3X$	generic designation for this protein family
$\alpha_3(3,5)F_2Y$	$\alpha_3Y$ containing 3,5-difluorotyrosine
APB buffer	sodium acetate, potassium phosphate, sodium borate buffer

<b>ATP</b>	adenosine 5'-triphosphate
<b>CDP</b>	cytidine 5'-diphosphate
$E^{\circ}$	formal reduction potential
$E_{\text{peak}}$	voltammetry peak potential
$E_{\text{for}}$	$E_{\text{rev}}$ and $E_{\text{net}}$ , peak potential of the forward, reverse and net current in SWV
$E_{\text{step}}$	staircase base potential in SWV
$E_{\text{SW}}$	square-wave amplitude
<b>EPR</b>	electron paramagnetic resonance
<b>ET</b>	electron transfer
<b>f</b>	square-wave frequency
<b>F<sub>n</sub>Y (n = 2, 3)</b>	di- and tri-substituted fluorotyrosines
<b>F<sub>n</sub>Y. RS</b>	fluorotyrosine aminoacyl-tRNA synthetase
<b>pK<sub>app</sub></b>	apparent pK <sub>a</sub>
$I_{\text{for}}$	$I_{\text{rev}}$ and $I_{\text{net}}$ , forward, reverse and net current in SWV
<b>PCET</b>	proton-coupled electron transfer
<b>PGE</b>	pyrolytic graphite edge
<b>RNR</b>	ribonucleotide reductase
<b>SWV</b>	square-wave voltammetry
<b>TPL</b>	tyrosine phenol lyase
<b>Y•</b>	tyrosine radical

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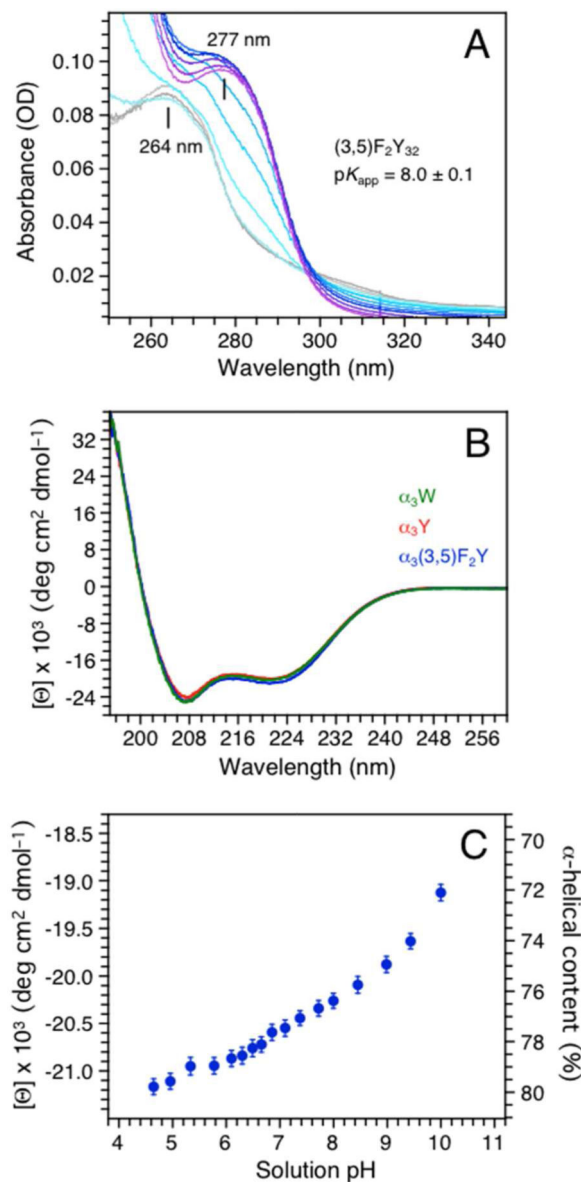
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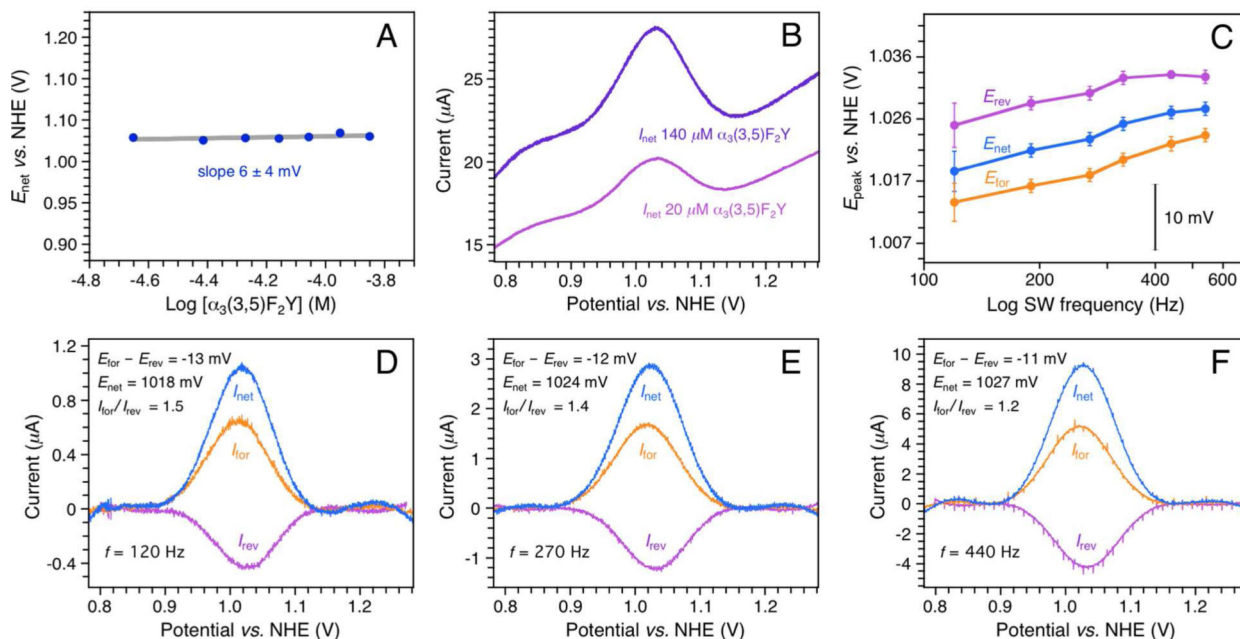
*GSR(1)–VKALEEKVKALEEKVKA–LGGGGR–IEELKKKX(32)EELKKKIEE–  
LGGGGE–VKKVEEEVKKLEEEIKK–L(65)*

**Scheme 1.**



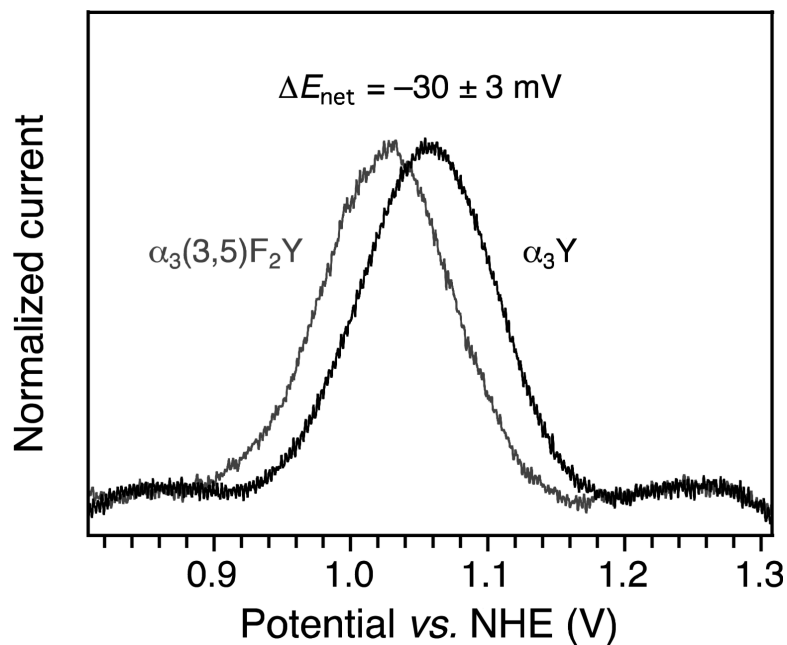
**Figure 1.**

(A) A pH titration of  $\alpha_3(3,5)F_2Y$  monitored by UV-Vis absorption spectroscopy. Spectra were collected at pH 5.72 (gray), 6.38, 6.84, 7.49, 8.06, 8.53, 8.96, 9.31, 9.59, 10.02 and 10.42 (purple). (B) Far-UV CD spectra of  $\alpha_3(3,5)F_2Y$  (blue),  $\alpha_3Y$  (red) and  $\alpha_3W$  (green) in 20 mM sodium acetate, pH 5.62  $\pm$  0.01. The single aromatic residue in each protein is fully protonated at this pH. The CD spectra are displayed in units of mean residue molar ellipticity ( $[\Theta]$ ) obtained by:  $[\Theta] = \theta_{obs}(10^6/Cln)$  where  $\theta_{obs}$  is the observed ellipticity in mdegrees (spectrometer raw signal),  $C$  the protein concentration in  $\mu$ M,  $l$  the cuvette path length in mm, and  $n$  the number of amino-acid residues (65).  $[\Theta]_{222}$  equals  $-20.0 \pm 0.8 \times 10^3$  deg cm<sup>2</sup> dmol<sup>-1</sup> at pH 5.6 for  $\alpha_3(3,5)F_2Y$  (Table 1). (C) Changes in the mean residue molar ellipticity ( $[\Theta]$ ) and the corresponding %  $\alpha$ -helical content of  $\alpha_3(3,5)F_2Y$  between pH 4.7 and 10.



**Figure 2.**

SWV analysis of the Y-O•/Y-OH redox system in  $\alpha_3(3,5)F_2Y$ . **(A)**  $E_{net}(270\text{ Hz})$  as a function of  $[\alpha_3(3,5)F_2Y]$ , pH  $5.53 \pm 0.11$ .  $E_{net}$  is independent of the protein concentration, which is consistent with benign protein/working electrode interactions. **(B)** Representative net voltammograms ( $I_{net}$ ) collected at the upper (140  $\mu\text{M}$ , purple) and lower (20  $\mu\text{M}$ , magenta) limit of the  $[\alpha_3(3,5)F_2Y]$  data series shown in panel A. The Faradaic response is optimal in this protein concentration range. **(C)** Peak potential of the forward ( $E_{for}$ ), reverse ( $E_{rev}$ ) and net ( $E_{net}$ )  $\alpha_3(3,5)F_2Y$  voltammograms as a function of the SW frequency ( $f$ ), pH  $5.70 \pm 0.02$ . **(D, E and F)** Background-corrected voltammograms from the data series shown in panel C. SWV settings: 20 mM APB, 75 mM KCl, PGE working electrode, temperature  $25^\circ\text{C}$ , step potential 0.15 mV, SW pulse amplitude 25 mV.



**Figure 3.**

$I_{\text{net}}$  of  $\alpha_3(3,5)F_2Y$  (grey) and  $\alpha_3Y$  (black) recorded at identical experimental conditions. SWV settings: 20 mM APB, 75 mM KCl, pH  $5.62 \pm 0.02$ , PGE working electrode, temperature  $25^\circ \text{C}$ , step potential 0.15 mV, SW frequency 190 Hz, SW pulse amplitude 25 mV.

Table 1

Properties of Y, 3,5-F<sub>2</sub>Y, α<sub>3</sub>Y and α<sub>3</sub>(3,5)F<sub>2</sub>Y

System	$\lambda_{max}$ (nm) / $\epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> )	p <i>K</i> <sub>a</sub> [p <i>K</i> <sub>app</sub> ]	Potential vs. NHE (mV) <sup>e</sup>	Ref.
Y(Ac/NH <sub>2</sub> ) <sup>a</sup>	275/1400 (Y-OH) 293/2420 (Y-O <sup>-</sup> )	10	<i>E</i> <sub>peak</sub> 830 (pH 7.0)	31
3,5-F <sub>2</sub> Y(Ac/NH <sub>2</sub> ) <sup>a</sup>	263/560 (Y-OH) 275/1830 (Y-O <sup>-</sup> )	7.0 ± 0.2	<i>E</i> <sub>peak</sub> 770 (pH 7.0)	31
α <sub>3</sub> Y	277/1500 (Y-OH, pH 5.4) 294/2430 (Y-O <sup>-</sup> , pH 13.4)	[11.3 ± 0.1]	<i>E</i> <sup>o'</sup> 1059 (pH 5.7) <i>E</i> <sup>o'</sup> 980 (pH 7.0)	10, 22, 23 this work
α <sub>3</sub> (3,5)F <sub>2</sub> Y	~264/n.d. (Y-OH) <sup>a</sup> ~277/n.d. (Y-O <sup>-</sup> ) <sup>a</sup>	[8.0 ± 0.1]	<i>E</i> <sup>o'</sup> 1026 (pH 5.7) <i>E</i> <sup>o'</sup> 952 (pH 7.0) <sup>f</sup>	this work

System	[ $\Theta$ ] <sub>222</sub> × 10 <sup>3</sup> (deg cm <sup>2</sup> dmol <sup>-1</sup> )	Helix (%) <sup>b</sup>	G (kcal mol <sup>-1</sup> ) <sup>g</sup>	Ref.
α <sub>3</sub> Y	-20.0 ± 0.8 (pH 5.6)	75 ± 3 (pH 5.6) <sup>c</sup> 74 ± 1 (4.5-10) <sup>d</sup>	-3.7 ± 0.1 (pH 5.0) -3.9 ± 0.1 (pH 5.5)	10, 22, this work
α <sub>3</sub> (3,5)F <sub>2</sub> Y	-20.9 ± 0.6 (pH 5.6)	79 ± 2 (pH 5.6) <sup>c</sup>	-3.7 ± 0.1 (pH 5.0) -3.7 ± 0.1 (pH 5.5)	this work

<sup>a</sup> Acetyl-tyrosinamide (Ac/NH<sub>2</sub>),  $\epsilon$  was not determined (n.d.).<sup>b</sup> Scaled relative to the [ $\Theta$ ]<sub>222</sub> of α<sub>3</sub>W (76 ± 1% α-helical, pH 4-10; 10, 25).<sup>c</sup> [Protein] determined by the Bradford assay.<sup>d</sup> [Protein] determined by UV absorption (10, 22).<sup>e</sup> Anodic peak potentials from irreversible differential pulse voltammograms (*E*<sub>peak</sub>; 10, 11, 31). The standard error in *E*<sup>o'</sup> is ± 4 mV.<sup>f</sup> Extrapolated from the pH 5.7 value and assuming the same pH dependence as observed for α<sub>3</sub>Y *E*<sup>o'</sup>(pH 5.7) and *E*<sup>o'</sup>(pH 7.0)(23).<sup>g</sup> From Fig. S5.