Site-Specific Incorporation of 3-Nitrotyrosine as a Probe of pK\(_{a}\) Perturbation of Redox-Active Tyrosines in Ribonucleotide Reductase

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Site-specific incorporation of 3-nitrotyrosine as a probe of $pK_a$ perturbation of redox-active tyrosines in ribonucleotide reductase

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Abstract

*E. coli* ribonucleotide reductase catalyzes the reduction of nucleoside 5′-diphosphates into 2′-deoxyribonucleotides and is composed of two subunits: α2 and β2. During turnover, a stable tyrosyl radical (Y·) at Y122-β2 reversibly oxidizes C439 in the active site of α2. This radical propagation step is proposed to occur over 35 Å, to use specific redox-active tyrosines (Y731 and Y730 in α2), and to involve proton-coupled electron transfer (PCET). 3-Nitrotyrosine (NO$_2$Y, $pK_a$7.1) has been incorporated in place of Y122, Y731 and Y730 to probe how the protein environment perturbs each $pK_a$ in the presence of the second subunit, substrate (S), and allosteric effector (E). The activity of each mutant is < 4 × 10$^{-3}$ that of the wt subunit. The [NO$_2$Y730]-α2 and [NO$_2$Y731]-α2 each exhibits a $pK_a$ of 7.8 – 8.0 with E and E/β2. The $pK_a$ of [NO$_2$Y730]-α2 is elevated to 8.2 - 8.3 in the S/E/β2 complex, while no further perturbation is observed for [NO$_2$Y731]-α2. Mutations in pathway residues adjacent to the NO$_2$Y that disrupt H bonding minimally perturb its $K_a$. The $pK_a$ of NO$_2$Y122-β2 alone or with α2/S/E is > 9.6. X-ray crystal structures have been obtained for all NO$_2$Y-α2 mutants (2.1 – 3.1 Å resolution), which show minimal structural perturbation compared to wt-α2. Together with the $pK_a$ of the previously reported NO$_2$Y356-β2 (7.5 in the α2/S/E complex, Yee, C. et al, *Biochemistry* 2003, 42, 14541-14552.), these studies provide a picture of the protein environment of the ground state at each Y in the PCET pathway and are the starting point for understanding differences in PCET mechanisms at each residue in the pathway.

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SUPPORTING INFORMATION PARAGRAPH (Word Style “TE_Supporting_Information”). SDS-PAGE of purified [NO$_2$Y]-α2s; absorption spectra of [NO$_2$Y]-α2s in their denatured state; absorption spectra of N-acetyl-3-nitrotyrosine amide (0.2 mM) in organic solvent; SDS-PAGE and ESI-QTOF-MS of β expressed in *E. coli* TOP10/pBAD-NrdB-CS(Y356Z)/pEVOL-NO$_2$Y; UV-vis absorption spectra of [NO$_2$Y122]-β2 with α2/ATP/CDP; pH titration curves of [NO$_2$Y]-α2s; the environment of Y122 in the crystal structure of wt-β2; $pK_a$s of α-, m- and n-nitroenol in solution; primers used for cloning and site-directed mutagenesis; crystallographic data collection and refinement statistics; the purity of [NO$_2$Y]-α2s and β2; $\lambda_{max}$ of N-acetyl-3-nitrotyrosine in organic solvent with 1% triethylamine; Stability of diferric cluster of met-[NO$_2$Y122]-β2 in basic conditions. This material is available free of charge via the Internet at http://ubs.acs.org.
Introduction

Class Ia ribonucleotide reductases (RNRs) play a crucial role in DNA replication and repair catalyzing the reduction of nucleoside 5'-diphosphates (NDPs) to 2'-deoxyribonucleoside 5'-diphosphates (dNMPs).\(^1\)\(^-\)\(^3\) α catalyzing the reduction of nucleotide 5′-deoxyribose (dNDPs).\(^1\)\(^-\)\(^3\) α catalyzing the reduction of nucleotide 5′-deoxyribose (dNDPs).\(^1\)\(^-\)\(^3\) This E. coli RNR consists of two homodimeric subunits: α2 and β2. α2 houses the binding sites for substrates (S) and effectors (E), where S is UDP, CDP, ADP or GDP and E is ATP, dATP, TTP or GTP that control the specificity and rate of nucleotide reduction. β2 harbors the radical initiator, a diferric tyrosyl radical (Y\(_{122}^•\)) cofactor, which reversibly and transiently oxidizes C\(_{439}\) in the active site of α2. This thyl radical initiates nucleotide reduction. The crystal structure of E. coli α2\(^{24,5}\) and of β2\(^{6,7}\) have been independently solved. A crystal structure of the class Ib RNR from Salmonella typhimurium containing both subunits has also been reported at 4.5 Å resolution and may be indicative of an intermediate in the formation of the active RNR complex that has remained elusive.\(^8\) A docking model by Uhlin and Eklund using the structures of α2 and β2 has been generated based on shape complementarity, in which the stable Y\(_{122}^-\) in β2 is > 35 Å from the C\(_{439}\) in α2. This distance is too large for electron tunneling, given the enzyme’s turnover number of 1-10 s\(^{-1}\).\(^{10,11}\) These observations led to the proposal that the radical propagation proceeds by a hopping mechanism through conserved aromatic amino acid residues located in α2 and β2 (Fig. 1).\(^{4,12-15}\) The present paper reports the site-specific incorporation of the unnatural amino acid 3-nitrotyrosine (NO\(_2\)Y) in place of Y\(_{356}\) in β2 and Y\(_{731}\) and Y\(_{730}\) in α2. The studies with these constructs reveal that NO\(_2\)Y is an excellent probe of how the protein environment modulates the pK\(_a\) of the henol, which is important for thinking about the different mechanisms of proton-coupled electron transfer (PCET) between Y\(_{122}\) and C\(_{439}\).

Evidence for the radical propagation pathway shown in Fig. 1 has come from a number of different experiments. Initially, site-directed mutagenesis of each of the conserved aromatic residues on the proposed pathway demonstrated that each is necessary for RNR activity.\(^{13,16,17}\) However, the inactivity of these mutants recluded further mechanistic investigation. Strong support for the redox role of Y\(_{356}\) in β2 was obtained by site-specific incorporation of a variety of tyrosine analogs in place of this residue by the use of expressed synthetase (RS)/tRNA method.\(^23\) The NH\(_2\)Y residues of [NH\(_2\)Y\(_{731}\)]\(^-\)α2 and [NH\(_2\)Y\(_{730}\)]\(^-\)α2 in the presence of β2/S and β2/S/E are oxidized to an aminotyrosyl radical (NH\(_2\)Y\(_{122}\)) in a kinetically competent fashion suggesting that these Y residues are redox active. Recent

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studies, in which NH$_2$Y was incorporated “off-pathway”,24 in conjunction with the Y$_{731}$NH$_2$Y-$\alpha$2 and Y$_{730}$NH$_2$Y-$\alpha$2 studies suggest that radical propagation occurs by a specific pathway.

Several additional results support the proposal of a co-linear PCET (also called hydrogen atom transfer, HAT) mechanism within $\alpha$. The first is our studies with a photoreactive-Y$_{356}$R2C19mer peptide complexed to $\alpha$. This 20mer-peptide, identical to the 20 C-terminal amino acid residues of $\beta$ including Y$_{356}$, contains an appended photo-oxidant at its N-terminus. It forms a complex with $\alpha$2 and makes deoxynucleotides subsequent to light-mediated oxidation of Y$_{356}$, thus acting as a competent surrogate for the entire $\beta$2.25/26 The results with the $\alpha$ mutant Y$_{730}$F and different photooxidants supports the proton-dependent hopping mechanism.26/27 The second is the observation that [NH$_2$Y$_{731}$]- and [NH$_2$Y$_{730}$]-$\alpha$2 and, more recently, [NH$_2$Y$_{356}$]-$\beta$2 (E. C. Minnihan, and J. Stubbe, unpublished results) are active in nucleotide reduction. An energetic analysis of the possible mechanisms of oxidation of C$_{439}$-$\alpha$2 by NH$_2$Y$_{730}$-$\alpha$2 supports a hydrogen atom transfer mechanism.23

The experimental data obtained thus far suggest different mechanisms of PCET occur at different residues within the pathway, which in part is a reflection of differences in the protein environment at each site.4/6/14 In order to think about the mechanism of PCET at each site (Fig. 1), a knowledge of the H bonding interactions and $pK_a$ perturbations of each Y within the pathway is necessary.14/15 Given the number of enzymes that utilize a specific pathway.

As a demonstration of its utility, we previously introduced NO$_2$Y in place of Y$_{356}$ in $\beta$2 and showed that its $pK_a$ was perturbed 0.2 pH units in the presence of $\alpha$2/ADP/dGTP, relative to the $pK_a$ of N-acetyl-3-nitrotyrosine amide in aqueous solution.29 We proposed that NO$_2$Y would be an excellent probe for a several reasons, albeit with some caveats. First, its $pK_a$ of 7.1 is in the middle of the pH range where most proteins, including RNR, are stable. Second, the absorption maximum for its phenol and phenolate at 360 nm and 424 nm, respectively, possess moderately large extinction coefficients and absorption features removed from the absorption maximum for its phenol and phenolate at 360 nm and 424 nm, respectively. Finally, in the case of probing the p$_K_a$ of a single Y in a protein with many Ys is of general interest.

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In this work, we have used the NO$_2$Y-RS/tRNA pair, recently evolved by the Chin and Mehl groups,35 to successfully incorporate NO$_2$Y in place of Y$_{731}$ and Y$_{730}$ of $\alpha$2 and Y$_{122}$ of $\beta$2. The EPL method, used to incorporate NO$_2$Y in place of Y$_{356}$, is not applicable for Y$_{731}$ and Y$_{730}$, due to the large size of the protein and the buried nature of the backbone of these residues. The $pK_a$ of each of these NO$_2$Ys (Fig. 1) has been determined in the presence of a single protein subunit and in the $\alpha$2$\beta$2/E and $\alpha$2$\beta$2/E/S complexes. Together with the previously determined $pK_a$ of NO$_2$Y at 356-$\beta$2,29 the data suggest distinct, position-dependent $pK_a$s with minimal perturbations for the three Ys (730, and 731-$\alpha$2 and 356-$\beta$2).
that form transient radicals, compared to the large perturbation at the stable Y· site, Y\textsubscript{122}. In addition, substrate binding perturbs the pK\textsubscript{a} of NO\textsubscript{2}Y only at 730. Additional mutations adjacent to the NO\textsubscript{2}Y substitution were introduced at positions 731, 730, and 439 in α2, to investigate the effect of the H bonding interactions (Fig. 1) on the pK\textsubscript{a}. Minimal effects were observed. Finally, the crystal structures for all the NO\textsubscript{2}Y-α2s have been obtained to 2.1 - 3.1 Å resolution and show minimal structural perturbations relative to the wt-α2 crystallized under identical conditions. The NO\textsubscript{2} group is planar with the phenol ring of residue 730 and shows two orientations (planar and perpendicular to the phenol ring) in residue 731. The results of NO\textsubscript{2}Y incorporation in these three positions provide the foundation for thinking about differences in mechanism of PCET at different residues in the pathway. The results further demonstrate that NO\textsubscript{2}Y is a sensitive reporter on protein environment.

Materials and methods

Luria Bertani (LB) medium, BactoAgar, 100 mm Petri dish plates were obtained from Becton-Dickinson. NO\textsubscript{2}Y, M9 salts, ampicillin (Amp), L-arabinose (L-Ara), chloramphenicol (Cm), all amino acids, ATP, 2'-deoxyguanine 5'-triphosphate (dGTP), cytidine 5'-diphosphate (CDP), adenine 5'-diphosphate (ADP), NADPH, ethylenediamine tetraacetic acid (EDTA), Bradford Reagent, Sehadex G-25, phenylmethanesulfonyl fluoride (PMSF) and streptomycin sulfate were purchased from Sigma-Aldrich. Isoproyl-β-thiogalactopyranoside (IPTG), dithiothreitol (DTT), cytidine 5′-diphosphate (CDP), adenine 5′-diphosphate (ADP), NADPH, ethylenediamine tetraacetic acid (EDTA), Bradford Reagent, Sehadex G-25, phenylmethanesulfonyl fluoride (PMSF) and streptomycin sulfate were purchased from Sigma-Aldrich. Isoproyl-β-thiogalactopyranoside (IPTG), dithiothreitol (DTT), PMSF and T4 DNA ligase were from Promega, pBAD/Myc-His A, E. coli DH5α and TOP10 competent cells, pCR2.1-TOPO, bacterial alkaline phosphatase and oligonucleotides were from Invitrogen. Calf-intestine alkaline phosphatase (CIAP, 20 U/μL) was from Roche. Ncol, Xhol, Sall, BgII, Ndel and PstI were from New England Biolabs. The purification of E. coli thioredoxin36 (TR, 40 units/mg), E. coli thioredoxin reductase37 (TRR, 1400 units/mg), and wild-type (wt) ββ238 (6200-7200 nmol/min/mg, 1-1.2 radicals per ββ) and wt α239 (2300-2600 nmol/min/mg) have previously been described. The concentrations of α2 and [NO\textsubscript{2}Y]-α2s were determined using ε\textsubscript{280nm} = 189 mM\textsuperscript{-1} cm\textsuperscript{-1}.40 The concentrations of ββ and [NO\textsubscript{2}Y\textsubscript{122}]-ββ were determined using ε\textsubscript{280nm} = 131 mM\textsuperscript{-1} cm\textsuperscript{-1}.40 The concentrations of apo-[NO\textsubscript{2}Y\textsubscript{122}]-ββ were determined using ε\textsubscript{280nm} = 120 mM\textsuperscript{-1} cm\textsuperscript{-1}.41 Glycerol minimal media leucine (GMML)42 contains final concentrations of 1% (v/v) glycerol, 1×M9 salts, 0.05% (w/v) NaCl, 1 mM MgSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, and 0.3 mM L-leucine. RNR assay buffer consists of 50 mM HEPES, 15 mM MgSO\textsubscript{4}, 1 mM EDTA, pH 7.6. UV-vis absorption spectra and the spectrophotometric assays were carried out using the Cary 3 UV-vis Spectrophotometer (Varian, Walnut Creek, CA). The Ultramark EX Microlate Imaging System (BioRad) was used to determine A\textsubscript{280nm} for protein, A\textsubscript{340nm} for diferric cluster and NO\textsubscript{2}Y phenol, A\textsubscript{490nm} for NO\textsubscript{2}Y phenolate of fractions after column chromatography. All DNA sequences were confirmed by the MIT Biopolymers Laboratory. PCR was carried out using PfufUltraII polymerase (Stratagene) according to the manufacturer’s protocol. The annealing temperature and number of cycles varied are described individually. For site-directed mutagenesis, PCR was performed for 18 cycles with annealing temperature of 55°C, followed by DpnI digestion of methylated template plasmid. The primers used for PCR are summarized in Table S2. pSUP-3NT/835 was kindly supplied by Dr. J. W. Chin (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) and Dr. R. A. Mehl’s lab (Franklin & Marshall College, Lancaster) and pEVOL43 was a kind gift of Dr. P. G. Schultz (The Scripps Research Institute, San Diego).

Construction of pBAD-nrdA-Y\textsubscript{730Z} and BAD-nrdA-Y\textsubscript{731Z}

nrdA was amplified by PCR using pMJ1-nrdA44 as a template with primers, nrdA-f and nrdA-r (Table S2) that harbor Ncol and Xhol restriction sites, respectively. Introduction of the Ncol site changed the first base of the second codon, A to G, which mutated the second
Amino acid, Asn to Asp. PCR was carried out as described above for 40 cycles with an annealing temperature of 58°C. The product was digested with Ncol and Xhol and subcloned into pBAD/MycHis-A to give pBAD-nrdA. The TAG codon (Z) was inserted at 730 or 731 using primers Y730Z-f and Y730Z-r for 730, and Y731Z-f and Y731Z-r for 731 (Table S2). The mutation accompanied by Ncol site introduction in the first PCR was repaired using primers nrdA-repair-f and nrdA-repair-r (Table S2) to give pBAD-nrdA-Y730Z and pBAD-nrdA-Y731Z.

Construction of EVOL-NO$_2$Y

pEVOL43 carries two copies of an RS gene, one with SalI and BglII sites and the other with NdeI and SstI sites in the upstream and downstream regions of the gene, respectively. SalI and BglII sites were introduced into the 3-nitrotyrosine RS (NO$_2$Y-RS) gene by PCR using pSUP-3NT/835 as a template and primers RS-f and RS-r (Table S2). PCR was carried out for 30 cycles with an annealing temperature of 55°C. Adenine was then added to the 3′-terminus of the PCR product for TA ligation by incubation with GO-Tag Hot start polymerase (Promega) in the presence of 0.2 mM dATP at 72°C for 30 min. The product was ligated with PCR2.1-TOPO (Invitrogen) following the manufacturer’s protocol. The resulting plasmid was first digested with BglII and a 3.9 kb DNA fragment containing NO$_2$Y-RS gene was isolated and further digested with SalI. The second NO$_2$Y-RS gene with NdeI and PstI sites was prepared by digesting pSUP-3NT/8 with NdeI and PstI. The two NO$_2$Y-RS gene fragments were sequentially introduced into pEVOL. Initially, the NO$_2$Y-RS gene flanked by NdeI/PstI was ligated into pEVOL. The NO$_2$Y-RS gene flanked with BglII/SalI was then ligated into the new construct to give pEVOL-NO$_2$Y.

Expression of [NO$_2$Y$_{730}$]-α2 and [NO$_2$Y$_{731}$]-α2

E. coli TOP10 was transformed with pSUP-3NT/835 (Cm$^R$) and pBAD-nrdA-Y$_{730}$Z and grown overnight on LB agar plates. All growths were carried out in the presence of Cm (25 mg/L) and Amp (100 mg/L). A single colony was picked and grown in LB to saturation (~16 h). Two mL of this solution was diluted into 200 mL LB in a 500 mL baffled flask and grown at 37°C until saturation. A portion of this culture (100 mL) was then inoculated into a fermenter containing 10 L of GMML medium (pH 6.8) supplemented with Cm (25 mg/L), Amp (100 mg/L), 17 amino acids (Glu, Gln, Asp, Asn, Lys, Val, Arg, Leu, His, Ile, Ala, Pro, Trp, Gly, Met, Thr, Ser, 0.2 g/L each), 1-glucose 0.05% (w/v), Asp 0.25% (w/v), L-ara 0.1% (w/v) and 1X heavy metal solution. The heavy metal stock solution (1000X) contained the following per L: 45 500 mg of Na$_2$MoO$_4$·2H$_2$O, 250 mg of CoCl$_2$, 175 mg of CuSO$_4$·5H$_2$O, 1 g of MnSO$_4$·H$_2$O, 8.75 g of MgSO$_4$·7H$_2$O, 1.25 g of ZnSO$_4$·7H$_2$O, 1.25 g of FeCl$_2$·4H$_2$O, 2.5 g of CaCl$_2$·2H$_2$O and 1 g of H$_2$BO$_3$, dissolved in 1 M HCl. NO$_2$Y (2 mM) was added at the beginning of the culture and growth was continued for 30 h (OD$_{600}$ = 2.6 – 2.8) at which time the cells were harvested by centrifugation, frozen in liquid N$_2$ and stored at −80°C. Typically, 5-6 g of wet cell paste/L were obtained. Expression of [NO$_2$Y$_{731}$]-α2 and all double mutants were carried out in an identical fashion using the appropriate pBAD constructs.

Purification of [NO$_2$Y$_{730}$]-α2 and [NO$_2$Y$_{731}$]-α2

[NO$_2$Y]-α2s were typically purified from 60-80 g of cell paste. All purification steps were carried out at 4°C. The cell paste was re-suspended in 5 volumes of buffer A (50 mM Tris pH 7.6, 1 mM EDTA, 1 mM PMSF and 5 mM DTT). The cells were lysed by a single passage through a French pressure cell operating at 14 000 psi. After removal of cell debris by centrifugation (20000g, 20 min, 4°C), DNA was precipitated by dropwise addition of 0.2 volumes of buffer A containing 8% (w/v) streptomycin sulfate. The mixture was stirred for an additional 15 min, and the precipitated DNA was removed by centrifugation (20000g, 20
Solid (NH$_4$)$_2$SO$_4$ (3.9 g per 10 mL of supernatant) was then added over 15 min (66% saturation). The solution was stirred for an additional 20 min and the precipitated protein was isolated by centrifugation (20000g, 20 min, 4°C). The precipitate was then added over 15 min (66% saturation). The solution was stirred for an additional 20 min and the precipitated protein was isolated by centrifugation (20000g, 20 min, 4°C). The pellet was dissolved in a minimal volume of buffer A and desalted using a Sephadex G-25 column (5 cm × 50 cm, 1 L). The desalted protein was loaded onto DEAE Fast Flow column (7 cm × 14 cm, 500 mL), which had been equilibrated in buffer A. The column was washed with 2 L of buffer A containing 50 mM NaCl followed by a gradient (1 L × 1 L) of 50-500 mM NaCl in buffer A. Fractions (10 mL each) containing [NO$_2$Y]-α2 were judged by SDS-PAGE and differences of phenolate absorption (A$_{490nm}$) at pH 7.6 and 9.0. [NO$_2$Y]-α2 was typically eluted at 210-250 mM NaCl. Pooled fractions were then directly loaded onto a dATP affinity column46 (1.5 cm × 4 cm, 100 mL), which had been equilibrated in buffer A. The column was washed with 10 column volumes of buffer A. [NO$_2$Y]-α2 was then eluted with buffer B (50 mM Tris pH 7.6, 5% glycerol, 1 mM EDTA, 15 mM MgSO$_4$, and 5 mM DTT) supplemented with 5 mM ATP and 5 mM DTT. Fractions containing NO$_2$Y-α2, judged by SDS-PAGE, were pooled and concentrated by ultrafiltration (Amicon YM-30, Millipore) and loaded onto Q-sepharose HP (Sigma-Aldrich, 3 × 5 cm, 35 mL), which had been equilibrated in buffer C (50 mM Tris pH 8.0, 5% glycerol, 1 mM EDTA, 15 mM MgSO$_4$, and 5 mM DTT). The column was washed with buffer C containing 50 mM NaCl and 1 mM ATP. ATP was added to revent equilibration between the monomer and dimer of α, which results in the protein eluting over a larger volume. The protein was eluted using a gradient (300 mL × 300 mL) of 50-500 mM NaCl in the same buffer. Fractions containing [NO$_2$Y]-α2 (typically, 160 - 210 mM NaCl), judged by SDS-PAGE, were pooled and concentrated by ultrafiltration as above and desalted using a Sephadex G-25 column (1.1 × 100 cm, 100 mL) for 4°C. The resultant protein solution was concentrated to ~90 μM, frozen in liquid N$_2$, and stored at −80°C. Typically, 1-3 mg of [NO$_2$Y]-α2s were obtained per g of wet cell paste.

Generation and expression of [NO$_2$Y$_{122}$]-β2

A TAG codon was introduced by PCR using pBAD-nrdB-NS547 as a template and primers, Y122Z-f and Y122Z-r (Table S2). NrdB encoded in pBAD-nrdB-NS5 has a StrepII tag (WSHPQFEK) at its N-terminus followed by a 5 amino acid linker (SLGGH). The resulting plasmid, pBAD-nrdB-NS5-Y$_{122}$Z (Amp$^R$), was introduced into E. coli TOP10 together with pEVOL-NO$_2$Y (Cm$^R$) and grown overnight on LB agarose plates. All growths were carried out in the presence of Cm (25 mg/L) and Amp (100 mg/L). A single colony was picked and grown in 5 mL LB to saturation (~16 h). Two mL of this solution was diluted into 200 mL LB in a 500 mL baffled flask and grown at 37°C until saturation (~10 h). A portion of this culture (100 mL) was then inoculated into a fermenter containing 10 L of the 17 amino acids supplemented GMML medium described above. NO$_2$Y (2 mM) was added at the beginning of the culture and growth was continued for 15 h (OD$_{600}$ = 1.5-1.8) at which point the cells were harvested by centrifugation, frozen in liquid N$_2$, and stored at −80°C. Typically, 2-3 g of wet cell paste/L were obtained.

Purification of [NO$_2$Y$_{122}$]-β2

[NO$_2$Y$_{122}$]-β2 was purified from 30-40 g wet cell paste. Although the [NO$_2$Y$_{122}$]-β2 is strep-tagged, the standard protocol38 for β2 purification was used for large scale preparations due to the poor binding of tagged [NO$_2$Y$_{122}$]-β2 to Streptactin sepharose (IBA, St. Louis, MO). All steps were carried out at 4°C. The cell paste was re-suspended in 5 volumes of buffer D (50 mM Tris pH 7.6, 5% glycerol and 0.5 mM PMSF). The cells were lysed by a single passage through a French pressure cell operating at 14,000 psi. Fe$^{II}$(NH$_4$)$_2$SO$_4$ and sodium ascorbate (5 mg each per g cell paste) were added to the lysate and stirred for 10 min. After removal of cell debris by centrifugation (20000g, 20 min,
4 °C). DNA was precipitated by drowise addition of 0.2 volumes of buffer D containing 6% (w/v) streptomycin sulfate. The mixture was stirred for an additional 15 min, and the precipitated DNA was removed by centrifugation (20000g, 20 min, 4°C). Solid (NH₄)₂SO₄ (3.9 g or 10 mL of supernatant) was then added over 15 min (66% saturation). The solution was stirred for an additional 20 min and the precipitated protein was isolated by centrifugation (20000g, 20 min, 4°C). The pellet was dissolved in a minimal volume of buffer D and desalted using a Sephadex G-25 column (5 cm × 50 cm, 1 L). The desalted protein was loaded onto DEAE Fast Flow column (5 cm × 13 cm, 250 mL), which had been equilibrated in buffer D. The column was washed with 1.5 L of buffer D containing 100 mM NaCl followed by a gradient (1 L × 1 L) of 100-500 mM NaCl in buffer D. Fractions containing [NO₂Y₇₃₀]-β2 were judged by SDS-PAGE and A₃₄₀nm, the absorption feature of diferric cluster and 3-nitrophenol. [NO₂Y₁₂₂]-β2 typically eluted at 190 – 220 mM NaCl. The resulting fractions were then diluted two-fold using buffer D and were loaded onto a Q-Sepharose fast flow column (4.5 cm × 10 cm, 150 mL), which had been equilibrated in buffer D. The column was washed with 1.5 L of buffer D containing 150 mM NaCl followed by a gradient (1 L × 1 L) of 150-500 mM NaCl in buffer D. Fractions containing [NO₂Y₁₂₂]-β2 (220-250 mM NaCl), judged by SDS-PAGE, were pooled and concentrated by ultrafiltration (Amicon YM-30, Millipore) to ~500 μL. Typically, 0.4 mg of [NO₂Y₁₂₂]-β2 was obtained per g of wet cell paste. The iron content was determined by the ferrozine assay.48

For activity assays, Strep-Tactin sepharose (IBA, St. Louis, MO) was used to remove contaminating wt-β2 from [NO₂Y₁₂₂]-β2. Typically 4-5 g of cell paste was used. Cell lysis, streptomycin sulfate precipitation and ammonium sulfate precipitation were carried out as described above. After removal of ammonium sulfate using Sephadex G-25, the desalted protein was loaded onto Strep-tactin sepharose column (1 cm × 5 cm, 4 mL), which had been equilibrated in buffer D. The column was washed with buffer D and [NO₂Y₁₂₂]-β2 was eluted with 2.5 mM desthiobiotin in buffer D. Typically, 0.4 mg of [NO₂Y₁₂₂]-β2 was obtained per g of wet cell paste. The decreased yield is due to a low affinity of [NO₂Y₁₂₂]-β2 for the resin. Protein concentration and iron content were determined as described above.

**Generation and expression of double mutants: [NO₂Y₇₃₀]-α2(Y₇₃₁F), [NO₂Y₆₇₀]-α2(Y₇₃₁A), [NO₂Y₆₇₀]-α2(C₄₃₉S), [NO₂Y₆₇₀]-α2(C₄₃₉A), and [NO₂Y₇₃₁]-α2(Y₇₃₀F)**

Mutations were introduced by PCR as described above with primers in Table S2. pBAD-nrdA-Y₇₃₀Z was used as a template for [NO₂Y₆₇₀]-α2(Y₇₃₁F), [NO₂Y₆₇₀]-α2(Y₇₃₁A), [NO₂Y₆₇₀]-α2(C₄₃₉S) and [NO₂Y₆₇₀]-α2(C₄₃₉A), and pBAD-nrdA-Y₇₃₁Z was used as a template for [NO₂Y₇₃₁]-α2(Y₇₃₀F). PCR products were amplified in *E. coli* DH5α and their DNA sequences were confirmed. Expression and purification of these proteins were carried out as described above for NO₂Y-α2 single mutants.

**Determination of protein purity and NO₂Y incorporation**

The purity of each [NO₂Y]-α2 and [NO₂Y]-β2 was determined from SDS-PAGE and Coomassie staining using Quantity One software (BioRad). The amount of NO₂Y incorporated into α2 or β2 was determined by its phenolate absorption subsequent to its incubation in 6 M guanidine in 50 mM TAS pH 9.0 at room temperature for 1 h. The measurement was repeated in triplicate. N-acetyl-3-nitrotyrosine amide, prepared as previously described29 and recrystallized from methanol, was used as a standard. The solvent was removed in vacuo over P₂O₅ for 18 h, until no further changes in weight were
observed. The extinction coefficient of N-acetyl-3-nitrotyrosine amide was determined by weight and UV-vis spectrum.

**K_d for the interaction of β2 and [NO_2Y]-α2s in the presence of CDP/ATP by the comitative inhibition assay.**

The assay mixture in a final volume 300 μL consisted of 0.15 μM β2, 1 mM CDP, 1.6 mM ATP, 50 μM TR, 1 μM TRR, 0.2 mM NADH in RNR assay buffer. The reaction was initiated by adding a mixture of 0.3 μM wt α2 and variable amounts of [NO_2Y]-α2s (0, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 μM) and the A_{340nm} (ε = 6.22 mM⁻¹ cm⁻¹), corresponding to NADPH consumption, was monitored at 25°C. The data were fit to eq. 1.

\[
[\text{NO}_2Y - \alpha 2]_{\text{bound}} = \left[\alpha 2\beta 2\right]_{\text{max}} \times [\text{NO}_2Y - \alpha 2]_{\text{free}} / (K_d + [\text{NO}_2Y - \alpha 2]_{\text{free}})
\]

where [NO_2Y-α2]_{bound} is the concentration of the NO_2Y-α2β2 complex, [α2β2]_{max} is the concentration of the wt-α2β2 complex in the absence of [NO_2Y-α2], and K_d is the dissociation constant of [NO_2Y]-α2 from β2. [α2β2]_{max} was 0.09 μM under these assay conditions. Each data point represents an average of three independent measurements. The K_d for the subunit interaction between α2 and [NO_2Y_{122}]-β2 was measured in the identical manner using 0.15 μM wt α2 and 0.3 μM wt β2.

**Spectrophotometric and Radioactive Activity Assays**

The spectrophotometric and radioactive RNR assays were performed as described. Typically, [NO_2Y]-α2s (1 or 2 μM), wt β2 (2, 5 or 10 μM) was incubated with CD (1 mM), ATP (3 mM) in the presence of TR, TRR and NADH in RNR assay buffer. [5-^3H]-CDP (5150 - 5230 cpm/nmol, ViTrax, Placentia, CA) was used in the radioactive assay.

**Determination of the pK_a of NO_2Y in [NO_2Y]-α2s and [NO_2Y_{356}]-β2 in the presence of E, E/β2 (or α2) and E/β2(or α2)/S**

To determine the pK_a of NO_2Y in the α2 mutants, the absorption spectra from 250 to 700 nm were determined at 25 °C at each pH. Each step in the titration was carried out in a separate cuvette. Good’s buffers (50 mM, MES (pH 6.0 – 6.8), HEPES (pH 7.0 – 8.0), and TAPS (pH 8.2 – 9.2) contained 1 mM EDTA and 15 mM MgSO_4. Each buffered solution contained: [NO_2Y]-α2 (7.5 μM) and E (ATP or dGTP at 1 mM and 0.1 mM, respectively), E/β2 (7.5 μM), or E/β2/S (CDP or ADP at 1 mM). The pH was redetermined after mixing all components. To compensate for differences in baseline absorption, all spectra were zeroed at 700 nm. All titrations were conducted in triplicate. Analysis of the titration data was carried out using eq.2.

\[
A = (\epsilon H K_d [H^+] + \epsilon^-) [\text{NO}_2Y - \alpha] / (K_d [H^+] + 1)
\]

where A is the absorbance of the phenolate, ε^- is the extinction coefficient of phenolate at its λ_{max}, and εH is the extinction coefficient of the phenol at the same wavelength as the phenolate λ_{max}. The data from the lot of A vs pH was fit to eq.2 using Kaleidagraph and non-linear least squares curve fitting to determine εH, ε^- and pK_a.

[NO_2Y_{356}]-β2, previously prepared by EPL29 was used to determine the pK_a in the complex with α2/ATP/CDP. The titration was carried out as described above, in the presence of E/α2 (1 mM ATP, 7.5 μM α2), or E/α2/S (1 mM CDP). The data were fit to eq.2 to determine the pK_a.
Incorporation of NO

Results

pH titration of NO\textsubscript{2}Y in [NO\textsubscript{2}Y\textsubscript{122}]-β2

The absorption spectrum of [NO\textsubscript{2}Y\textsubscript{122}]-β2 was determined between pH 7.0 and 10.6 at 25 °C as above. For a pH higher than 9.0, CHES (pH 9.0 – 10.0) and CAPS (pH 10.3 – 10.6) were used. Titrations were carried out with [NO\textsubscript{2}Y\textsubscript{122}]-β2 (7.5 μM), with [NO\textsubscript{2}Y\textsubscript{122}]-β2 (7.5 μM)/α2 (7.5 μM)/ATP (1 mM)/CDP (1 mM), or with apo-[NO\textsubscript{2}Y\textsubscript{122}]-β2 (7.5 μM). The pH was measured after mixing all components. To compensate for differences in baseline absorption, all spectra were zeroed at 700 nm. Ao-[NO\textsubscript{2}Y\textsubscript{122}]-β2 was prepared using hydroxyquinoline as a chelator in the presence of 1 M imidazole.41

Crystallization, data collection and refinement

Wt-α2 and [NO\textsubscript{2}Y]-α2s were co-crystallized with a 20mer peptide4 that corresponds to the last 20 amino acids of β2. Crystals were grown for approximately one week at 4 °C using the hanging drop vapor diffusion method in EasyXtal Tool plates (QIAGEN). Each drop consisted of 2 μL α-mutant (8-9 mg/ml) and peptide (30 mg/ml) and 2 μL of a solution containing 1.5 M Li\textsubscript{2}SO\textsubscript{4}, 2 mM DTT and 25 mM sodium citrate (pH 6). The final pH of each drop was 6.0-6.5. The crystals were quickly washed in a 1.5 M Li\textsubscript{2}SO\textsubscript{4} solution containing 20% ethylene glycol, mounted in fiber loops and flash-frozen in liquid N\textsubscript{2}.

All sets of data were collected at 100 K at the European Synchrotron Radiation Facility stations (Grenoble, France). The crystals are of the rhombohedral space group R3\textsubscript{2} with hexagonal axes approximately 225, 225 and 337 Å, containing 3 molecules per asymmetric unit. Due to the freezing of the crystals, the cells are slightly smaller than the previous data of wt-α2 collected at 4 °C.4 Y\textsubscript{730F}-α2 coordinates (DPB ID, 1R1R) were used as an initial model in refinement of the wt-α2 data (2.3 Å resolution). This wt-α2 structure was then used as a starting model for all [NO\textsubscript{2}Y]-α2s. To verify the positions of modifications/mutations, difference maps between the new wt-α2 structure and each [NO\textsubscript{2}Y]-α2 structure were inspected after the first restrained cycle of refinement. Table S3 summarizes the details of data collection and refinement.

Results

Incorporation of NO\textsubscript{2}Y into α2

The Chin and Mehl groups35 recently reported the evolution of a suppressor tRNA/RS pair for site-specific incorporation of NO\textsubscript{2}Y into superoxide dismutase (SOD) expressed in E. coli. Initially their construct pSUP-3NT/8, containing the genes for both the tRNA and the RS was used in combination with pTrc containing nrdA (the gene for RNR α) with the TAG codon in place of the tyrosine codon at 730 or 731. pTrc plasmid has recently been successfully used by us to incorporate NH\textsubscript{2} into the same positions of α2,23 Expression was carried out in GMML medium or in the minimal medium used for NO\textsubscript{2}Y incorporation into SOD.35 Under these conditions, however, only very weak expression of truncated α2 was observed. In an effort to enhance expression of α, we cloned nrdA into pBAD/MycHis-A to generate pBAD-nrdA and attempted expression in richer medium.50 E. coli TOP10 cells were transformed with pSUP-3NT/8 and pBAD-nrdA-Y\textsubscript{730}Z or pBAD-nrdA-Y\textsubscript{731}Z and grown in GMML medium containing NO\textsubscript{2}Y, D-glucose, L-ara and 17 proteinogenic amino acids excluding Cys, Tyr and Phe. Under these conditions, protein expression was robust, giving rise to predominantly truncated α and a small amount of full-length protein (Fig. 2a, left gel). Fortunately, most of the truncated protein was insoluble, and thus full-length α was successfully isolated by our standard procedure for α purification which uses a dATP affinity column,46 followed by an additional Q Sepharose column (Fig. S1). Typically 2.8 mg of [NO\textsubscript{2}Y\textsubscript{730}]-α2 and 1.0 mg of [NO\textsubscript{2}Y\textsubscript{731}]-α2 are isolated per g of cell paste. More recently we have used the newly constructed pEVOl-NO\textsubscript{2}Y (see below) that results in higher expression levels of full length [NO\textsubscript{2}Y\textsubscript{730}(731)]-α2 (Fig. 2a, right gel).
The purity of \([\text{NO}_2\text{Y}_{730}]\)-\(\alpha\)2 and \([\text{NO}_2\text{Y}_{731}]\)-\(\alpha\)2 was determined to be ~95% and ~80%, respectively, based on the intensities of the Coomassie stained protein bands on SDS-PAGE gel (Fig. S1 and Table S4). At pH 9.2, these mutants exhibit a phenolate \(\lambda_{\text{max}}\) of 442 nm and 437 nm, respectively, which is red-shifted 18 nm and 13 nm from the phenolate \(\lambda_{\text{max}}\) of \(N\)-acetyl-3-nitrotyrosine amide\(^2\) (424 nm) in the same buffer (Fig. 3a). To calculate the extent of \(\text{NO}_2\text{Y}\) incorporation into \(\alpha\), each \([\text{NO}_2\text{Y}]\)-\(\alpha\)2 was denatured in 6 M guanidine HCl in 50 mM TASA pH 9.2. Both denatured mutants exhibited a phenolate \(\lambda_{\text{max}}\) of 435 nm, identical to that of the \(N\)-acetyl-3-nitrotyrosine amide in the same buffer (Fig. S2).\(^5\) The extinction coefficient (\(A_{435}\)) of \(N\)-acetyl-3-nitrotyrosine amide in 6 M guanidine (pH 9.2) was determined to be 4890 M\(^{-1}\)cm\(^{-1}\).\(^5\) By measuring \(A_{435}\) of the denatured \([\text{NO}_2\text{Y}]\)-\(\alpha\)2s, the concentration of \(\text{NO}_2\text{Y}\) in each mutant was determined. These numbers allowed the determination of the purity of \([\text{NO}_2\text{Y}_{730}]\)-\(\alpha\)2 and \([\text{NO}_2\text{Y}_{731}]\)-\(\alpha\)2 to be 93 ± 3% and 79 ± 3%, respectively, which correlate well with the purity of \(\alpha\)2 determined from SDS-PAGE analysis (Table S4), suggesting that the impurity is associated with other proteins and that incorporation of \(\text{NO}_2\text{Y}\) into both \([\text{NO}_2\text{Y}]\)-\(\alpha\)2s, is high (98 ± 2%).

**Incorporation of \(\text{NO}_2\text{Y}\) into \(\beta\)2**

In contrast with the results using pSUP-3NT/8 for the incorporation of \(\text{NO}_2\text{Y}\) into \(\alpha\), efforts to express full-length \(\beta\) with \(\text{NO}_2\text{Y}\) at 122 were unsuccessful, despite detection of substantial amounts of truncated protein. Recently, the Schultz group has developed a new plasmid, pEVOL, in which aminoacyl-RS expression is controlled by the strong ara inducible promoter, araBAD.\(^4\) pEVOL has been demonstrated to improve suppression relative to other constructs in a number of cases.\(^4\) Thus, the \(\text{NO}_2\text{Y}\)-RS was cloned into pEVOL resulting in pEVOL-\(\text{NO}_2\text{Y}\) and used to examine \(\text{NO}_2\text{Y}\) incorporation in place of \(\text{Y}_{122}\) in \(\beta\). Successful expression of full-length \(\beta\)2 was observed when the protein was expressed in the amino acid supplemented minimal medium using pEVOL-\(\text{NO}_2\text{Y}\) and pBAD-\(\text{nrdB}\)-NS5-\(\text{Y}_{122}\)Z (Fig. 2b). pBAD-\(\text{nrdB}\)-NS5-\(\text{Y}_{122}\)Z encodes \(\beta\) with Strep\(\text{II}\) tag and a 5 amino acid linker (SLGGH) at the N-terminus and a TAG codon (\(\text{Z}\)) at position 122. We have previously confirmed that the N-terminal Strep-tag does not affect the RNR activity.\(^4\) In contrast, Strep\(\text{II}\)-tagged \(\beta\)2 will be referred to as \(\beta\)2. Attempts to purify \([\text{NO}_2\text{Y}_{122}]\)-\(\beta\)2 with the Strep\(\text{II}\) tag and a 5 amino acid linker (SLGGH) at the N-terminus and a TAG codon (\(\text{Z}\)) at position 122. We have previously confirmed that the N-terminal Strep-tag does not affect the RNR activity.\(^4\) Strep\(\text{II}\)-tagged \(\beta\)2 was purified by our standard procedure for non-tagged \(\beta\)2 using anion exchange column chromatography. Typically 6-7 mg \([\text{NO}_2\text{Y}_{122}]\)-\(\beta\)2 per g cell paste with 90% purity based on SDS-PAGE gel analysis, was obtained (Fig. 2b, Table S4).

As isolated, the absorption spectrum of the purified \([\text{NO}_2\text{Y}_{122}]\)-\(\beta\)2 showed broad features between 325 and 400 nm and no sharp feature associated with a Y· at 410 nm (Fig. 3b). The ferrozine assay revealed 2.9 ± 0.2 Fe/\(\beta\)2, suggesting that \([\text{NO}_2\text{Y}_{122}]\)-\(\beta\)2 has a diferric cluster (met-form). Thus, if \(\text{NO}_2\text{Y}\)- is formed, it is short-lived. Incorporation of \(\text{NO}_2\text{Y}\) was confirmed to be ~98% as described above for \(\alpha\) (Table S4). Subtraction of the absorption spectrum of the diferric cofactor cluster (normalized for the iron content) from that of \([\text{NO}_2\text{Y}_{122}]\)-\(\beta\)2 gave a spectrum with a \(\lambda_{\text{max}}\) at 363 nm (\(e = 3400 ± 240\) M\(^{-1}\)cm\(^{-1}\), Table 1, Fig. 3b), consistent with a phenol form of \(\text{NO}_2\text{Y}\).

Finally, incorporation of \(\text{NO}_2\text{Y}\) in place of \(\text{Y}_{356}\) was also attempted. Since the truncated \(\beta\) was soluble, C-terminal Strep\(\text{II}\)-tagged \(\beta\)47 was used to facilitate the separation of full-length \(\beta\) from truncated \(\beta\). The use of pEVOL-\(\text{NO}_2\text{Y}\) and pBAD-\(\text{nrdB}\)-Y\(_{356}\)Z resulted in expression of full-length \(\beta\) that was not observed when the protein was expressed in the absence of \(\text{NO}_2\text{Y}\) (Fig. S3). However, the purified full-length \(\beta\) did not exhibit the phenolate absorption at pH 9.2 under native or denaturing conditions, suggesting the absence of \(\text{NO}_2\text{Y}\) incorporation. An ESI-MS analysis showed the purified \(\beta\) has a molecular mass 15 Da larger than the wt protein (Fig. S4), which corresponds to an addition of an NH\(_2\) group. No signal corresponding to \([\text{NO}_2\text{Y}_{356}]\)-\(\beta\)2 was observed. Previous workers have reported that \(\text{NO}_2\text{Y}\)
can be reduced to NH$_2$Y by heme proteins in the presence of thiols or ascorbic acid.52 Since residue 356 is surface exposed, it is likely to have been reduced in *E. coli* cells.

**Determination of the $K_d$ for Subunit interactions**

The $K_d$ for subunit interactions in wt-RNR is weak [0.06 - 0.2 μM in the presence of CDPaneutral and ATP,49 (A. Q. Hassan and J. Stubbe, unpublished results) and 0.4 μM in the absence of nucleotides]54]. Perturbation of subunit interactions by NO$_2$Y incorporation was thus a concern as 731 is thought to reside at the subunit interface. Thus, the $K_d$ was determined by the procedure of Climent et al.49, using the [NO$_2$Y]-α2s and [NO$_2$Y]$_{Y2}$-β2 as a competitive inhibitor of wt-α2 and β2 interactions. This method gave a $K_d$ of 1.32 ± 0.10 μM, 0.51 ± 0.07 μM and 0.40 ± 0.06 μM for [NO$_2$Y]$_{Y30}$-α2, [NO$_2$Y]$_{Y731}$-α2 and [NO$_2$Y]$_{Y122}$-β2, respectively (Table 2). Since the formation of the phenolate of NO$_2$Y could perturb subunit interactions, the $K_d$ was also determined at pH 6.8 where the phenol is completely protonated. The observed $K_d$ for [NO$_2$Y]$_{Y731}$-α2 (0.53 ± 0.07 μM) is essentially the same as that at pH 7.6. In general, the interactions are weaker than those observed in wt RNR. Unexpectedly, the $K_d$ of [NO$_2$Y]$_{Y731}$-α2 is higher than that of [NO$_2$Y]$_{Y2}$-α2, even though Y$_{Y31}$-α2 is closer to the subunit interface.4 This knowledge facilitated design of studies, p$K_a$ determination and activity assays, where the subunits need to be associated.55

**Catalytic activity of NO$_2$Y-α2s: levels of endogenous wt-α**

The genes for RNR are essential and therefore expression of [NO$_2$Y]-β or [NO$_2$Y]-α mutants in *E. coli* is accompanied by expression of small amounts of the endogenous wt-subunit. The purification protocol for [NO$_2$Y]$_{Y355}$-β2 made by EPL ensured the removal of wt-β and thus a lower limit of detection of deoxynucleotide formation for this mutant of < 1/10$^4$ that of wt-β2 was set.29 The absence of activity was expected based on the experimentally measured differences in peak potentials of the NO$_2$Y and Y of 210 mV (pH 7.6)29 and our previous studies with F$_y$Y (n = 2-4) at the same position, which suggested that when the F$_y$Y was more difficult to oxidize than Y by 200 mV, RNR was inactive.20

Activity of [NO$_2$Y]$_{Y122}$-β2 with an N-terminal Strep-tag and without a tag, purified by affinity chromatography and by conventional chromatography methods, respectively, was determined. In the former case, the [NO$_2$Y]$_{Y122}$-β2 had activity 1/250 and in the latter case < 1/10$^4$ of the wt-β2 (Table 3). Thus, [NO$_2$Y]$_{Y122}$-β2 is inactive.

The activity assays of [NO$_2$Y]$_{Y30}$- and [NO$_2$Y]$_{Y731}$-α2, Table 3, revealed activity levels of 0.5 – 1.5% for [NO$_2$Y]$_{Y30}$-α2 (1/200 to 1/100) to 0.4 - 2.9% for [NO$_2$Y]$_{Y731}$-α2 (1/250 to 1/30) that of wt-α2. Three arguments support our conclusion that the observed activity is predominantly associated with endogenous wt-α. First there is a rough correlation between the levels of expression of the mutants and their activity (Table 3): the higher the yield of the mutant protein, the lower the activity. Expression of [NO$_2$Y]$_{Y731}$-α, for example, with two different suppression constructs gives rise to activity that varies 5 fold. Second, a number of double mutants, described below, with NO$_2$Y and a block (he) in the ET pathway, all have activities in the range of 1 to 3% (1/30 to 1/100) of wt. The Phe mutants themselves have been deemed inactive with similar levels of activity.13 Third, the activity measured for [NO$_2$Y]-α2 assayed at 2 μM is independent of the concentration of β2 (2 μM, 5 μM or 10 μM) used in the assay mixture. Based on the $K_d$ for subunit interactions, the RNR activity would have been expected to increase with the increasing concentrations of β2 due to increased α2β2 complex formation.55

Finally, we have recently developed an affinity chromatography method to remove endogenous levels of wt-α2 from [NH$_2$Y]-α2s by constructing an N-terminally tagged α into which the unnatural amino acid is incorporated (E. C. Minnihan, and J. Stubbe,
unpublished results). Use of this construct to generate [NO\textsubscript{2}Y\textsubscript{730}]-α2 gave protein of 92% purity and activity 0.5% that of wt-α2 at pH 7.6. The activity is also 0.50 ± 0.02% that of wt-α at pH 6.5 and 8.6. If the mechanism of oxidation of C\textsubscript{439} involves hydrogen atom transfer, then at pH 8.6 the relative activity should be reduced to <25% of that at pH 6.8, as the NO\textsubscript{2}Y is predominantly the phenolate at pH 8.6 (see titration data below and Table 4). From the arguments presented above we have assumed that all the [NO\textsubscript{2}Y]-α2s and β2s are inactive and that the detected activity is associated with endogenous wt-α or with the infidelity of the RS associated with mischarging the tRNA with Y.

**Determination of the pK\textsubscript{s} of [NO\textsubscript{2}Y]-α2s**

The ability to form [NO\textsubscript{2}Y]-α2β2 complexes incapable of turnover has enabled us to determine the pK\textsubscript{s} of NO\textsubscript{2}Y at positions 730 and 731 in α2 in the presence S (ADP or CDP) and S/E air (CDP/ATP or ADP/dGTP). As noted above, S and E are critical as they trigger the active conformation for PCET. The pH measurements of the α2β22 complex were performed between pH 6.0 – 9.2 with [NO\textsubscript{2}Y]-α2 and β2 in which 65% of [NO\textsubscript{2}Y\textsubscript{730}]-α2 and 77% of [NO\textsubscript{2}Y\textsubscript{731}]-α2 are in a complex based on the K\textsubscript{d} (Table 2). An additional experiment was carried out with 92% complex formation with similar results. The errors in the pK\textsubscript{s} values were ± 0.05 pH units. The protein stability was also ascertained at the two pH extremes (6.0 and 9.2) by incubating the wt RNR at these pH for 15 min followed by an activity assay at pH 7.6. In each case 95% of the activity was retained. The results of a typical pH titration in the presence of E are shown in Fig. 4. As the pH increases, the absorption of the phenol decreases concomitant with an increase in the absorption of the phenolate with an isosbestic point at 393 nm. Plots of the intensity of the phenolate absorption vs pH are shown in Fig. 5. The data is best fit to eq.229 for a species undergoing a single deprotonation. The pK\textsubscript{s} of NO\textsubscript{2}Y in each [NO\textsubscript{2}Y]-α2 are summarized in Tables 4A and 4B and can be compared to the pK\textsubscript{s} of NO\textsubscript{2}Y in the C-terminal 20mer peptide of β2.29 The pK\textsubscript{s} of [NO\textsubscript{2}Y\textsubscript{730}]-α2 with E is 7.9 and 7.8 for ATP and dGTP, respectively. These pK\textsubscript{s} remain unchanged upon addition of β2. Addition of S to the [NO\textsubscript{2}Y\textsubscript{730}]-α2/E/β2 complex, either a purine or a pyrimidine, increased the pK\textsubscript{s} by 0.4 pH units. The pK\textsubscript{s} of NO\textsubscript{2}Y at 731 is similar to 730 when titrated with E or E/β2, however, no further change is observed on S addition.

The extinction coefficients for the phenolate of each NO\textsubscript{2}Y in α2 were also determined by fits to eq. 2 and are 6400 ± 250 and 5200 ± 300 M\textsuperscript{-1}cm\textsuperscript{-1} for [NO\textsubscript{2}Y\textsubscript{730}]-α2 and [NO\textsubscript{2}Y\textsubscript{731}]-α2, respectively (Table 1). These values are larger than those determined for N-acetyl-3-nitrotyrosine amide under the same conditions (λ\textsubscript{max} 424 nm, ε\textsubscript{424nm} 4610 M\textsuperscript{-1}cm\textsuperscript{-1}). The extinction coefficients of phenols were also calculated from the absorbance at λ\textsubscript{max} at pH 6.0 (Table 1). The differences in extinction coefficients and shifts in λ\textsubscript{max} of the mutants are reporting on their surrounding environment, but are difficult to interpret. To obtain insights about the effects of surrounding environment, the absorption spectra of N-acetyl-3-nitrotyrosine amide were determined in various organic solvents with 1% triethylamine (Fig. S5, Table S5). Red-shifted phenolate absorptions (440 – 458 nm) were observed in non-protic polar solvents (DMSO, DMF and acetonitrile). In contrast, in protic solvents (water, ethanol and methanol), the phenolate absorbs at shorter wavelength, 418 – 424 nm. Thus, the red shifts of NO\textsubscript{2}Y observed at 731 and 730 may suggest a less protic environment. π-π stacking interactions and NO\textsubscript{2} group orientation, however, can also affect the phenolate absorption, and theoretical studies using structures described below are ongoing to better understand the environment of each Y.

**pH titration of [NO\textsubscript{2}Y]-β2**

The pH titration of [NO\textsubscript{2}Y\textsubscript{122}]-β2 was carried out between pH 7.0 – 10.0 (Fig. 6a) with the spectra from pH 7.6 - 8.6 being identical. At pH 9.0, a decrease in A\textsubscript{360nm} is observed
without a corresponding increase in the region of the phenolate, 420 - 450 nm. At pH > 9.2, further decrease in absorption in the 320 – 360 nm region occurs along with an increase in the phenolate region, although no isosbestic point is apparent. The unusual absorption spectra at pHs greater than 9 for [NO₂Y₁₂₂]-β2 may be due to the decomposition of the diferric cluster that generates apo-β2 which then allows titration of NO₂Y₁₂₂. Quantitation of iron in [NO₂Y₁₂₂]-β2 at pH 10.0, for example, showed complete iron loss (Table S6) and the phenolate absorption observed is close to that predicted from the pKₐ of this residue in apo-β2 (see below). Thus, the pKₐ of the iron loaded form (met-form) cannot be directly determined. Since the spectra between pH 7.6 - 8.6 are identical, and 10 % of phenolate would result in a detectable change in absorbance (0.007, assuming an ε = 4200 M⁻¹cm⁻¹), a lower limit of detection of the pKₐ of the phenol can be set at 9.6. The pKₐ perturbation of NO₂Y₁₂₂ is thus greater than 2.5 pH units. Results from the titration of [NO₂Y₁₂₂]-β2 with wt-α2/E/S are similar to the titration without α2 (Fig. S6).

In contrast, a pH titration of NO₂Y was successfully carried out between pH 7.0 - 10.6 with apo-[NO₂Y₁₂₂]-β2 which does exhibit an isosbestic point (Fig. 6b). Titration above pH 10.8 was not possible due to protein precipitation. These data, fit to eq.2, gave a pKₐ of 9.8. This pKₐ is close to the lower limit of pKₐ for the met-form (9.6), indicating that the diferric cluster does not significantly lower the phenol pKₐ. The λmax and the extinction coefficient of phenolate were minimally perturbed from that of N-acetyl-3-nitrotyrosine amide in water (Table 1).

The pKₐ of [NO₂Y₃₅₆]-β2 was also revisited using ATP/CDP. [NO₂Y₃₅₆]-β2 prepared by EPL29 was used due the reduction of NO₂Y in 356 of β2 incorporated by the orthogonal tRNA/RS methodology. The pKₐ with ATP/α2 and ATP/α2/CDP are very similar to the previously determined pKₐ with dGTP/α2/ADP (Table 4c). For all the NO₂Y mutants, ATP/CDP and dGTP/ADP gave almost identical pKₐs.

### pKₐs of [NO₂Y]-α2s with a second mutation adjacent to NO₂Y in the PCET pathway

The crystal structure of GDP/TTP bound E. coli α2 indicates that the distance between the oxygens of the phenol of Y₇₃₁ and Y₇₃₀-α2 is 3.3 Å and the distance between the oxygen of Y₇₃₀-α2 and the sulfur of C₄₃₉-α2 is 3.4 Å.5 The distances indicate the possibility of pH bonding interactions between these species, which likely plays an important role in PCET. 56’59 The structure also suggests an unusual π–π stacking interaction of Y₇₃₁- and Y₇₃₀-α₂, which may also be of interest mechanistically, although the degree of stacking varies in recent structures of α from other sources.60’61 Removal of a H bonding interaction or the π stacking might perturb the pKₐ of the phenolic hydroxyl group. A variety of double mutants (Y to Phe/Ala and Cys to Ser/Ala) were successfully expressed and isolated. Their purity was similar to the corresponding parent single mutant (Table S4).

The λmax and ε of the phenolate, and the K₄ for subunit interactions for each double mutant were determined (Tables 1 and 2) and are similar to the parent single mutant. Conservative substitutions minimally perturbed the K₄, while the non-conservative substitution of Ala for C₄₃₉ or Y₇₃₁ in [NO₂Y₇₃₀]-α₂ resulted in large K₄ perturbations (Table 2). As noted above, all double mutants showed specific activities of 1-3% of wt-α2, similar to the single mutants and presumably associated with endogenous wt-α2.

The pKₐ of each double mutant was determined (Table 4A and 4B, Fig. S7). With [NO₂Y₇₃₀]-α₂, Y₇₃₁ was replaced with Phe and C₄₃₉ with Ala, mutations that disrupt the putative H bonding. C₄₃₉ was also replaced with Ser that could enhance H bonding. With the C₄₃₉A double mutant, the pKₐ was increased from 7.9 to 8.3/8.4 in the presence of E (ATP or dGTP) or Eβ2 and addition of S (CDP or ADP) again perturbed the pKₐ by 0.2 to 0.3 units. With Y₇₃₁F, the addition of S also elevated the pKₐ to 8.2. Only in the case of C₄₃₉S
did addition of substate aear to have no effect. However, as will be seen from the structures described in the following section, [NO$_2$Y$_{730}$]-α2/C$_{439}$S is the only mutant in which 439 is H bonded to the active site E$_{441}$ (Fig. 7d). Thus, we propose that S binding would disrupt this H bonding interaction and reset the pH of residue 730 with minimal perturbation in K$_d$ (Table 2) suggesting that the structure of the complex is perturbed by this non-conservative substitution.

Studies with [NO$_2$Y$_{731}$]-α2 and Y$_{731}$ replaced with an Ala were also examined to address the putative importance of π-π stacking interactions. The perturbations in pH for this mutant were the largest observed in any of the titrations, decreasing from 7.8/7.9 (single mutant) to 6.5 (double mutant) in the presence of E. The observed pHs are 0.6 units lower than the N-acetyl-3-nitrotyrosine amide. However, even in this case, the pH was substantially increased, from 6.5 to 6.8/7.0, on addition of S. This double mutant also exhibited the largest perturbation in K$_d$ (Table 2) suggesting that the structure of the complex is perturbed by this non-conservative substitution.

Titration studies were also carried out with [NO$_2$Y$_{731}$]-α2 and Y$_{356}$F-β2 and with the double mutant where Y$_{730}$ was replaced with F. In the former case, the pH was unaffected by S addition. However, the pH of the Y$_{730}$F double mutant decreased 0.5 units relative to the single mutant with E and E/α2 and increased back toward that of the single mutant pH on addition of S. The studies in sum suggest that S perturbs the pH at 730 with minimal effect at 731 and that the altered pH do not appear to be the result of increased H bonding by residues adjacent to the substitution.

Crystal structures of NO$_2$Y-α2s

Understanding the experimental data obtained with the unnatural amino acid probes in α and β requires structural information and computational efforts. As a first step toward structural characterization, crystals of wt and seven NO$_2$Y-α2 mutants were obtained in the presence of a 20mer peptide, identical to the C-terminal 20 amino acids of β, using crystallization conditions similar to those previously reported for wt-α2.4 The structure of wt-α2, isolated by methods similar to the NO$_2$Y-α2s, was solved by molecular replacement using Y$_{730}$F-α2 (DB ID, 1R1R). This new wt-α2 structure (2.3 Å resolution) was then used as the starting point to solve the NO$_2$Y-α2 structures, which were obtained and refined to 2.1 – 3.1 Å resolution. In all mutants, there are three α subunits in the asymmetric unit with two of the three subunits forming a dimer. The data collection methods and refinement statistics are summarized in Table S3. In general, the structures revealed minimal overall perturbation relative to the wt structure and the phenyl rings and OH groups of NO$_2$Ys superimposed well with the corresponding residues in the wt structure (Fig. 7). In all of the structures the distances between the oxygens of the two phenols (at 731 and 730) and of the phenol and the thiol (at 730 and 439) are very similar to the distances found in wt-α and are within H bonding distance in the former case and slightly longer than H bonding distance in the latter case.

The NO$_2$ group in all of the structures lies on the sterically less crowded side, the left side looking toward C$_{439}$ from Y$_{731}$ (Fig. 7a and 7b). At 730, the NO$_2$ group in all single and double mutants lies in the plane of the phenyl ring. No additional electron density is apparent in the vicinity of [NO$_2$Y$_{730}$]-α2 that can be associated with water. On the other hand, the NO$_2$ group at 731 has different orientations in each of the three αs in the asymmetric unit. In two of the αs, the NO$_2$ group is perpendicular to the plane of the phenyl ring, while in the remaining α, it is almost parallel to the ring. The oxygens of the NO$_2$ group have pH bonding interactions with H$_2$O(s), which are distinct within each α.
double mutant (Y730F) also has its NO₂ group perpendicular to the phenyl ring, but no waters are identifiable.

Finally a superposition of all the [NO₂Y730]-α structures (Fig. 7D) shows that residue 439 adots a different conformation in the serine mutant, in which the OH of serine appears to H bond to E441, a key residue in the active site involved in catalysis. The SH of Cys in the wt-α2, on the other hand, points toward residue 730. Thus, the structures in general suggest H bonding interactions are important in the PCET with C439 showing the greatest flexibility. The detailed analysis of the structures is ongoing and will be published subsequently. Ultimately, structures with S, E and β2 bound will be critical to understanding the role of H bonding interactions in the radical propagation process.

Discussion

Understanding of the radical propagation process in RNR requires an understanding of factors that control the different mechanisms of PCET in well-defined model systems where the rate constants for oxidation can be measured and the parameters that influence these rate constants such as H bonding, electronic effects, and the proton acceptor can be manipulated. Different types of models will be required to probe PCET within β and within α of RNR (Fig. 1). H bonded phenols, for example, have been designed to understand the oxidation of the YZ in photosystem II in which a histidine functions as the proton acceptor from YZ during its oxidation.68-69 phenols with amine and imidazole bases62-63/70 and carboxylates56/65/66 appended ortho to the phenol have been synthesized and structurally and spectroscopically characterized. The rate constants for oxidation and the deuterium kinetic isotope effects on oxidation have been measured as a function of temperature.56/62/65/66/70 The Hammarström model seems particularly appropriate for Y356 oxidation in RNR where E350 is proposed to be the initial proton acceptor (Fig. 1). However, appropriate models for the putative H bond network between Y731/Y730/C439, with no obvious base proximal to either Y, are not yet available.

As a starting point to understanding the PCET in RNR, we have chosen NO₂Y and NH₂Y as reporters on the H bonding interactions of each Y in the ground states and the transient Y· intermediate states in the radical propagation process, respectively. In the former case, we proposed that the phenol pKₐ might be altered by the protein environment on binding of the second subunit and/or S/E. In the latter case, ¹H and ²H ENDOR spectroscopy might allow direct observation of H bond interactions to the radical intermediates.71 The focus of this aer is NO₂Y.

To carry out these studies, the evolved tRNA/RS method pioneered by the Schultz lab42 was optimized to site-specifically incorporate high levels of NO₂Y in place of Y122, Y731 and Y730. Our efforts to insert NO₂Y in place of Y356 have thus far been unsuccessful due to reduction during the expression process. The inactivity of these mutants has allowed us to determine the pKₐ of each NO₂Y in the α2β2 complex in the presence of substrate (S) and effector (E), presumably in the “active” conformation required for PCET. Together with our previous pKₐ data for [NO₂Y356]-β2, the results presented show the power of site-specific incorporation of unnatural amino acids to measure individual pKₐs in a protein with 1135 amino acids. These measurements for four different NO₂Ys, provide an important step in understanding radical initiation in RNR.

Mechanistic insights

Our pH titration data has established that each Y in the pathway (Fig. 1) has distinct properties. The greatest pKₐ perturbations are observed with NO₂Y122-β2: elevations of 2.7 and >2.5 units in apo and met form, respectively. This Y is unique in that its oxidation
results in a stable Y· (t_{1/2} of 4 days at 4°C) and it is generated by the metal cluster. An explanation for its unusual stability is clear from a Comparison of X-ray crystal structures of met-β26 (β2 with Y· reduced-diferric cluster), apo-β272, and Mn2-β273 and results from single crystal high field EPR spectroscopy of the active diferric-Y·-β2.7 In the crystal structure of the class Ia Mn2-β273 the Y_{122}-OH is within H bonding distance to D_{84}, a carboxylic acid ligand to Mn1 (corresponds to Fe1 in Fig. 1b) of the cluster, that in turn may H bond with a H_2O (x = 1, 2) bound to the same Mn (Fig 1b). If similar interactions exist in the diferric cluster during radical propagation in the presence of α/S/E, then the close interaction of the phenol with the D_{84} carboxylate, would preclude its ionization, to prevent electrostatic repulsion with D_{84}. The pK_a of 9.8 for NO_2Y in apo-[NO_2Y_{122}]-β2 suggests that its environment, even in the apo form, is very hydrophobic (Q_{80}, I_{231}, I_{234}, F_{208}, L_{77}, F_{212}; Fig. S8). On the other hand, high field EPR spectroscopy of active β2 (Y·) reveals that this Y_{122} is no longer H bonded.7 Our current model is that only subsequent to S/E binding in α is PCET initiated and the Y· reoriented toward D_{84} so that it can pick a proton from the H_2O bound to Fe1 concomitant with its reduction by W_{48,12,14-15} Thus, the environment of the Y· is such that it is stable, unless a proton is also available. The elevated pK_a of NO_2Y observed in our titrations is consistent with concerted PCET mechanism for Y_{122} oxidation in which the proton donor is distinct from the reductant (W_{48}).

The pK_a of NO_2Y at 356 in β2 and 730 and 731 in α2 are perturbed to a much smaller extent than at 122 with perturbations increasing as the distance of the Y increases from Y_{122} (7.5, 7.9, 8.3 for NO_2Y 356, 731 and 730, respectively). The perturbation ranges from 0.4 to 1.2 units relative to NO_2Y within the 20mer-C-terminal peptide. These measurements demonstrate that Nature has pEVOLved a different type of environment for transient radical formation and a different mechanism for their oxidation. pK_a at each transient Y· site is discussed.

Studies in a variety of enzymes containing redox cofactors have established that binding of substrate provides a mechanism to perturb the reduction potential of the cofactor, and to facilitate the chemistry.75-76 We thus investigated whether the addition of the correct S/E air to RNR could alter the pK_a of each Y and provide insight about its oxidation mechanism. The pK_a of NO_2Y_{730} is uniquely increased by 0.4 units on addition of either a purine or pyrimidine substrate to the [NO_2Y_{730}]-α2/β2/E. The basis for this perturbation and the potential importance of the H bond network was investigated by titration studies with double mutants that have altered H bonding capabilities in residues adjacent to NO_2Y_{730}-α2. The parallel NO_2 group orientation relative to the plane of the phenyl group with all mutants suggests that relative pK_a's are informative. In all cases except C_{439}S, addition of substrate elevates the pK_a regardless of the H bonding interactions, consistent with an increased hydrophobic environment. No waters are identifiable in the region surrounding 730. The observations with the Ser mutant can also be rationalized as described in the Results. At present, we do not know if this small perturbation is mechanistically important or why the pK_a is increased. Finally, it is also likely that if H bonding is key to the mechanism of the thyl radical generation, it may be manifested predominantly in the transition states of the reaction and may not be readily apparent from this type of ground-state analysis.15-65

The two remaining Ys (Fig 1) are located at the subunit interface and unfortunately no structural information is available for the active α2β2 complex. Our studies, however, with 3,5-F_{2}Y at Y_{356} revealed that nucleotide reduction can occur through the phenolate form of this residue, consistent with non-obligate coupling of the electron and proton transfer and with the proton transfer occurring orthogonal to the ET pathway.21 The minimal perturbation of the NO_2Y pK_a at this position suggests it may be more solvent exposed in the complex than Y_{730}, which is also consistent with our recent studies using an environmentally sensitive fluorophore attached at 356.54 Our current hypothesis for PCET at this position is
that orthogonal PCET occurs with transfer of a proton to E$_{350}$ within the C-terminal tail of $\beta$. The importance of this residue in nucleotide reduction was demonstrated by previous studies of Climent et al.16 with a E$_{350}A$ mutant and our recent studies with E$_{350}Q$ mutant (unpublished results). As noted above, the model studies of Hammarström may thus provide a model of PCET at this center.56,66

The p$K_a$ of NO$_2$Y$_{731}$ and its minimal perturbation in double mutants with Y$_{356}F$ and Y$_{730}F$, suggests that a H bonding is again not observable at this position and that this residue may be solvent exposed. The structure of the single mutant shows that the NO$_2$ group is more flexible at this position with conformations both parallel and perpendicular to the plane of the phenyl ring and that it H bonds with specific water molecules that are distinct in each $\alpha$. Sequence analysis of conserved residues of RNR with the constraint that they reside within the subunit interface based on the docking model,4 reveal only E$_{350}$ and R$_{236}$ in $\beta$ and no interesting conserved residues in $\alpha$. Studies on R$_{265}$ mutants in mouse $\beta$, corresponding to the R$_{236}$ mutant in E. coli $\beta$, have been interpreted to support a role for this residue in the proton coupling in the propagation pathway.77 However, since R$_{265}E$ mutant exhibited 40% the activity of the wt enzyme, it is unlikely to function as a proton acceptor. Unfortunately, interactions that are proposed to be important in our models for radical propagation in RNR (Fig. 1)14-15 are not apparent from the p$K_a$ measurements. Our studies suggest that the p$K_a$s of the Ys that undergo transient oxidations during radical propagation in RNR do not appear to be significantly perturbed. Computational efforts are underway to use the structural information to understand the p$K_a$ perturbations.

Summary

Despite the prevalence of PCET in biological systems, p$K_a$ information of individual redox active residues in proteins is scarce. A general method for directly measuring the p$K_a$ of a single residue within a protein would be mechanistically useful.78 In this paper NO$_2$Y has been used as a probe to measure the p$K_a$ perturbations of the all redox-active Ys, 122- and 35674-$\beta_2$, 731- and 730-$\alpha_2$, in the radical propagation pathway in E. coli RNR. The crystal structures of the NO$_2$Y-$\alpha_2$ mutants have shown minimal structural perturbations. The pH titrations have shown position dependent p$K_a$ perturbations at these redox active Y sites with the largest and distinct perturbation at the stable tyrosyl radical site, Y$_{122}$-$\beta_2$. The study reveals distinct environments for the stable and transient Y·. The p$K_a$ information reported is essential to understand the mechanism of long range PCET in RNR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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This work was supported by the NIH grant GM29595 (to JS).

References


(74). pK_a of NO_2Y_356β2 was determined using the mutant prepared by EPL, which requires two additional mutations, V_353G and S354C, that reduce the activity to 25% of the wt activity.


Figure 1. Tyrosines responsible for the PCET in *E. coli* class I RNR. (a) Proposed PCET pathway. Red and blue arrows indicate orthogonal transfer of the electron and proton, respectively. The purple arrow indicates co-linear movement of the electron and proton. (b) Structure of the tyrosine-diferric-cluster. Distances in parentheses are those of the Mn$_2$-$\beta$.
Figure 2. SDS-PAGE of [NO$_2$Y$_{730}$]-α and [NO$_2$Y$_{122}$]-β. (a) SDS-PAGE of purified [NO$_2$Y$_{730}$]-α and whole cell lysate of *E. coli* expressing [NO$_2$Y$_{730}$]-α using pSUP-3NT/8 (left) and pEVOL-NO$_2$Y (right). Cells were grown in the presence or absence of NO$_2$Y as indicated. The position of protein bands for full-length α (85.6 kDa) and truncated α (82.2 kDa) are denoted by arrows. (b) SDS-PAGE of purified [NO$_2$Y$_{122}$]-β (45.0 kDa) and a whole cell lysate of *E. coli* TOP10/pEVOL-NO$_2$Y/pBAD-nrdB-NS5-Y$_{122}$Z grown in the absence and presence of NO$_2$Y as indicated. The truncated protein is 15.9 kDa and thus not observable in this gel (10% acrylamide).
Figure 3.
Absorption spectra of nitrophenolate feature of [NO$_2$Y]-α2s and nitrophenol feature of [NO$_2$Y$_{122}$]-β2. (a) [NO$_2$Y$_{730}$]-α2 (blue), [NO$_2$Y$_{731}$]-α2 (green) and N-acetyl-3-nitrotyrosine amide (yellow) in 50 mM TAPS (pH 9.0), 1 mM EDTA, 15 mM MgSO$_4$. Spectral intensities were normalized to NO$_2$Y concentration (15 μM) according to the purity of [NO$_2$Y$_{730}$]-α2 (92%), [NO$_2$Y$_{731}$]-α2 (79%) determined from $A_{435nm}$ in 6 M guanidine. (b) Red trace, absorption spectra of [NO$_2$Y$_{122}$]-β2 (2.9 Fe/β2, 15 μM) in HEPES (pH 7.6); blue trace, absorption spectrum after subtraction of the met-β2 spectrum (3.2 Fe/β2, 13.5 μM, the concentration of β2 was normalized for the iron content); black trace, absorption spectrum of N-acetyl-3-nitrotyrosine amide in 50 mM MES (pH 5.0) buffer.
Figure 4.
UV-vis absorption spectra of (a) [NO$_2$Y$_{730}$]-α2 (7.5 μM) and (b) [NO$_2$Y$_{731}$]-α2 (7.5 μM) at pH 6.0 (the pink trace), 7.4, 8.0, 8.6 and 9.2 (the blue trace) in the presence of 1 mM ATP. Loss of the phenol feature (360 nm) occurs concomitant with the formation of the phenolate feature (442 and 437 nm for [NO$_2$Y$_{730}$]-α2 and [NO$_2$Y$_{731}$]-α2, respectively) with increasing pH (pink to blue).
**Figure 5.**
Titration curves of (a) [NO$_2$Y$_{730}$]-α2 and (b) [NO$_2$Y$_{731}$]-α2 in the presence of 1 mM ATP (yellow diamonds), 1 mM ATP and 7.5 μM β2 (blue squares) and 1 mM ATP, 7.5 μM β2 and 1 mM CDP (red circles). Absorption at 442 nm and 437 nm were monitored for [NO$_2$Y$_{730}$]-α2 and [NO$_2$Y$_{731}$]-α2, respectively. Each data point represents an average of three replicates. Lines are from fits to eq. 2.
Figure 6.
UV-vis absorption spectra of (a) met-[NO₂Y₁₂2]-β2 at pH 7.1 (red), 7.6 (orange), 8.2 (yellow), 8.6 (dark green), 9.0 (light green), 9.2 (light blue), 9.5 (blue) and 10.0 (dark blue), and (b) apo-[NO₂Y₁₂2]-β2 at pH 7.6 (the pink trace), 8.4, 9.0, 9.6, 10.0, 10.3 and 10.6 (the blue trace).
Figure 7.
Crystal structures of the radical propagation pathway in NO$_2$Y-$\alpha$2 mutants. The crystals were grown at pH 6.0-6.5. Oxygens are colored in red, nitrogens in blue, and sulfurs in gold. Pathway residues 731, 730 and 439 are shown with sticks. The structure of (a) [NO$_2$Y$_{730}$]-$\alpha$2 and (b) [NO$_2$Y$_{731}$]-$\alpha$2; the dotted lines indicate the distances between the phenolic oxygens, and the phenolic oxygen of the 730 residue and the sulfur of C$_{439}$ with distance variations associated with the three subunits in the asymmetric unit. The surrounding residues are shown as spheres. (c) An overlay of the structures of wt-$\alpha$2 (green), [NO$_2$Y$_{730}$]-$\alpha$2 (orange), and [NO$_2$Y$_{731}$]-$\alpha$2 (yellow), generated using PyMOL 1.1 (DeLano Scientific LLC) software. Other residues shown are P$_{621}$, E$_{441}$, L$_{438}$, Y$_{413}$ and R$_{411}$. (d) An overlay of the structures of [NO$_2$Y$_{730}$]-$\alpha$2 and the double mutants C$_{439}$S, C$_{439}$A, Y$_{731}$A and Y$_{731}$F. Residues shown are (from the left) 441, 439, 730 and 731.
### Table 1
Extinction coefficients of NO$_2$Y phenol and phenolate in RNR

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<td>$\lambda_{\text{max}}$</td>
<td>$\varepsilon$ (M$^{-1}$cm$^{-1}$)$^b$</td>
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<tr>
<td>N-Ac-NO$_2$Y-NH$_2$</td>
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<td>3800 ± 170</td>
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<tr>
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<tr>
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<td>360 nm</td>
<td>3700 ± 220</td>
</tr>
<tr>
<td>[NO$<em>2$Y$</em>{730}$]$<em>2$-a2(C$</em>{439}$A)</td>
<td>360 nm</td>
<td>3400 ± 230</td>
</tr>
<tr>
<td>[NO$<em>2$Y$</em>{731}$]$<em>2$-a2(Y$</em>{730}$F)</td>
<td>360 nm</td>
<td>4000 ± 230</td>
</tr>
<tr>
<td>[NO$<em>2$Y$</em>{122}$]$_2$-β2 (met form)</td>
<td>363 nm</td>
<td>3400 ± 240$^c$</td>
</tr>
<tr>
<td>[NO$<em>2$Y$</em>{122}$]$_2$-β2 (apo form)</td>
<td>363 nm</td>
<td>3400 ± 240</td>
</tr>
</tbody>
</table>

$^a$ Extinction coefficients of phenolates were determined from pH titration curve fitting to eq. 2. The titration curves were generated by plotting absorbance at indicated phenolate $\lambda_{\text{max}}$ as a function of pH. Titration curves used are shown in Fig. 5 and Fig. S7.

$^b$ Extinction coefficients of phenol were calculated from the UV-vis spectrum at pH 6.0.

$^c$ The extinction coefficients of [NO$_2$Y$_{122}$]$_2$-β2 (met form) was determined from the absorption spectrum at pH 7.6 after subtracting a spectrum of wt met-β2 normalized by the iron content.
Table 2

$K_d$ of $[\text{NO}_2Y\alpha_2s$ with wt-$\beta_2$ or $[\text{NO}_2Y_{122}]\beta_2$ with wt-$\alpha_2$

<table>
<thead>
<tr>
<th></th>
<th>$K_d$ ($\mu$M)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{NO}<em>2Y</em>{730}]\alpha_2$</td>
<td>1.32 ± 0.10</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{731}]\alpha_2$</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{730}]\alpha_2(Y_{731}F)$</td>
<td>1.45 ± 0.14</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{730}]\alpha_2(Y_{731}A)$</td>
<td>2.71 ± 0.13</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{730}]\alpha_2(C_{499}S)$</td>
<td>1.28 ± 0.07</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{730}]\alpha_2(C_{499}A)$</td>
<td>2.07 ± 0.07</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{731}]\alpha_2(Y_{730}F)$</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>$[\text{NO}<em>2Y</em>{122}]\beta_2$</td>
<td>0.40 ± 0.06</td>
</tr>
</tbody>
</table>

$^aK_d$ was determined at pH 7.6 by using $[\text{NO}_2Y\alpha_2s$ or $[\text{NO}_2Y_{122}]\beta_2$ as competitive inhibitors of CDP reduction by $\alpha_2$, $\beta_2$, TR, TRR and NADPH.49
### Table 3

RNR activities of [NO₂·Y]-α2s and [NO₂·Y₁₂₂]-β2

<table>
<thead>
<tr>
<th></th>
<th>Radioactive RNR assay (% wt)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spectrophotometric RNR assay (% wt)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yield (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NO₂·Y₇₃₀]-α₂</td>
<td>1.1</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>His-[NO₂·Y₇₃₀]-α₂&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.5</td>
<td>5.8</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₁]-α₂</td>
<td>2.9</td>
<td>3.1</td>
<td>1</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₃]-α₂&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₀]-α₂(Y₇₃₁F)</td>
<td>2.5</td>
<td>2.7</td>
<td>0.8</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₀]-α₂(Y₇₃₁A)</td>
<td>1.8</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₀]-α₂(C₄₉₈S)</td>
<td>2.4</td>
<td>2.6</td>
<td>1.1</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₀]-α₂(C₄₉₈A)</td>
<td>2.7</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>[NO₂·Y₇₃₁]-α₂(Y₇₃₈F)</td>
<td>1.7</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>[NO₂·Y₁₂₂]-β₂&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.29</td>
<td>7</td>
</tr>
<tr>
<td>[NO₂·Y₁₂₂]-β₂&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>&gt; 0.01</td>
<td>&gt; 0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activities are reported as % activity of wt α2 (2500 nmol/min/mg) or wt β2 (6500 nmol/min/mg).

<sup>b</sup> Expressed using pEVOL-NO₂·Y.

<sup>c</sup> His-tagged NrdA purified with Ni-NTA column chromatography. See Experimental section for detail.

<sup>d</sup> Purified using DEAE and Q-sepharose.

<sup>e</sup> Purified using strep-Tactin sepharose.
### Table 4A

<table>
<thead>
<tr>
<th></th>
<th>[NO$<em>2$Y$</em>{730}$]-α2</th>
<th>[NO$<em>2$Y$</em>{730}$]-α2 (Y$_{731}$F)</th>
<th>[NO$<em>2$Y$</em>{730}$]-α2 (C$_{439}$S)</th>
<th>[NO$<em>2$Y$</em>{730}$]-α2 (C$_{439}$A)</th>
<th>[NO$<em>2$Y$</em>{730}$]-α2 (Y$_{731}$A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>7.9</td>
<td>7.9</td>
<td>8.3</td>
<td>8.3</td>
<td>6.5</td>
</tr>
<tr>
<td>ATP/β2</td>
<td>7.9</td>
<td>7.9</td>
<td>8.4</td>
<td>8.3</td>
<td>6.6</td>
</tr>
<tr>
<td>ATP/β2/CDP</td>
<td>8.3</td>
<td>8.2</td>
<td>8.3</td>
<td>8.5</td>
<td>7.0</td>
</tr>
<tr>
<td>dGTP</td>
<td>7.8</td>
<td>7.9</td>
<td>8.4</td>
<td>8.3</td>
<td>6.5</td>
</tr>
<tr>
<td>dGTP/β2</td>
<td>7.8</td>
<td>7.9</td>
<td>8.5</td>
<td>8.4</td>
<td>6.5</td>
</tr>
<tr>
<td>dGTP/β2/ADP</td>
<td>8.2</td>
<td>8.2</td>
<td>8.4</td>
<td>8.6</td>
<td>6.8</td>
</tr>
</tbody>
</table>

$pK_a$s were determined by fitting eq.2 to the pH titration curve generated by plotting phenolate absorption intensities as a function of pH. Measurements were carried out in the presence of $E$, $E/β2$ and $E/β2/S$ and performed in triplicate. Errors were ± 0.05 pH units.
Table 4B

pK$_a$ of [NO$_2$Y$_{731}$]-α2 and [NO$_2$Y$_{731}$]-α2(Y$_{730}$F)

<table>
<thead>
<tr>
<th></th>
<th>[NO$<em>2$Y$</em>{731}$]-α2</th>
<th>[NO$<em>2$Y$</em>{731}$]-α2 (Y$_{730}$F)</th>
<th>[NO$<em>2$Y$</em>{731}$]-α2 /Y$_{356}$F-β2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>7.9</td>
<td>7.4</td>
<td>-</td>
</tr>
<tr>
<td>ATP/β2</td>
<td>8.0</td>
<td>7.4</td>
<td>7.8</td>
</tr>
<tr>
<td>ATP/β2/CDP</td>
<td>8.0</td>
<td>7.7</td>
<td>7.8</td>
</tr>
<tr>
<td>dGTP</td>
<td>7.9</td>
<td>7.4</td>
<td>-</td>
</tr>
<tr>
<td>dGTP/β2</td>
<td>8.0</td>
<td>7.5</td>
<td>7.9</td>
</tr>
<tr>
<td>dGTP/β2/ADP</td>
<td>7.9</td>
<td>7.7</td>
<td>7.9</td>
</tr>
</tbody>
</table>

pK$_{a}$s were determined as described in the footnote of Table 4A. Titrations were carried out in the presence of E, E/β2 and E/β2/S and performed in triplicate. Errors were ± 0.05 pH units.
Table 4C

<table>
<thead>
<tr>
<th>[NO\textsubscript{2}Y\textsubscript{356}]\textsuperscript{-}β2</th>
<th>(pK_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>7.1\textsuperscript{a}</td>
</tr>
<tr>
<td>α, α2\textsuperscript{b}</td>
<td>7.2\textsuperscript{a}</td>
</tr>
<tr>
<td>ATP/α2</td>
<td>7.4</td>
</tr>
<tr>
<td>ATP/α2/CDP</td>
<td>7.5</td>
</tr>
<tr>
<td>dGTP/α2/ADP</td>
<td>7.3\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\([\text{NO}_2\text{Y}_{356}]\textsuperscript{-}β2\) was prepared by EPL and its \(pK_a\)\textsuperscript{a} were determined as described previously.\textsuperscript{29} Titrations were carried out in the presence of \(E/α2\) and \(E/α2/S\) and performed in triplicate. Errors were ± 0.05 pH units.

\(\textsuperscript{a}\)Values reported previously.\textsuperscript{29}

\(\textsuperscript{b}\)α is in equilibrium between monomer and dimer without nucleotides.\textsuperscript{40}