Supporting Information for

Site–Specific Insertion of 3–Aminotyrosine into Subunit α2 of *E. coli* Ribonucleotide Reductase: Direct Evidence for Involvement of Y_{730} and Y_{731} in Radical Propagation

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Cloning of pBAD–*nrdA* and Insertion of TAG codons. Vector pBAD–JYCUA was obtained from the Schultz lab (ref. 61, text). The *nrdA* gene was cloned from pMJ1–*nrdA* into pBAD–JYCUA using the NcoI and KpnI restriction sites by standard methods to yield pBAD–*nrdA*. Insertion of TAG codons at positions 730 and 731 was carried out as described for pTrc–*nrdA* using primers 3–6 (see Methods). The mutations were confirmed by sequencing the entire gene at the MIT Biopolymers Laboratory.

Generation of pMJ1–*nrdA*$_{730}$TAG and pMJ1–*nrdA*$_{731}$TAG. Vector pMJ1–*nrdA* has been reported before (ref. 60, text). Insertion of TAG codons at positions 730 and 731 was carried out as described for pTrc–*nrdA* using primers 3–6 (see Methods). The mutations were confirmed by sequencing the entire gene at the MIT Biopolymers Laboratory.

Attempts at Expression of $Y_{730}NH_2Y$–α2. In an effort to maximize production of α2 containing NH_2Y, without generation of NH_2Y•, growth conditions were examined at different temperatures, under aerobic and anaerobic conditions and with hydroxyurea in the media to reduce the Y_{122}• in wt β2.

In each case, a single colony of DH10B or BL21(DE3) *E. coli* cells was used to inoculate a 5 mL 2YT small culture. After saturation, this culture was diluted 100–fold into 2 × 100 mL GMML media. When $OD_{600\ nm}$ was ~0.6–0.8, and after the variations detailed below, one flask was supplemented with NH_2Y and DTT to final concentrations of 1 mM and 0.1 mM, respectively. The other growth served as the control. After 15 min, expression of NH_2Y–α2 was induced by addition of IPTG to both 100 mL cultures (or 0.2 % (w/v) L–arabinose with expression system (1) – see below). Small aliquots were removed from each flask after a defined time period (5–12 h) and expression of α2 assessed by SDS PAGE analysis in the presence and absence of NH_2Y/DTT.

When the effect of temperature on expression of $Y_{730}NH_2Y$–α2 was tested, growth conditions for the small culture were identical to those above. When $OD_{600\ nm}$ was ~0.6–0.8, the temperature setting was changed to 25 or 30°C. After 15 min, NH_2Y and DTT were added and the growth was continued as described above.

When the effect of Y_{122}•–β2 on expression of $Y_{730}NH_2Y$–α2 was tested, growth conditions for the small culture were identical to those described above. When $OD_{600\ nm}$ was 0.6–0.8, hydroxyurea was added to a final concentration of 65 mM. After 15 min, NH_2Y and DTT were added to final concentrations of 1 mM and 0.1 mM, respectively. After an additional 15 min, induction was carried out as above. Each hour after induction, the culture was supplemented with an additional 15 mM hydroxyurea.

When the effect of O_2 on expression of $Y_{730}NH_2Y$–α2 was tested, growth conditions for the small culture were identical to those described above. When the small culture was saturated, it was diluted 100–fold into a 250 mL 2YT medium containing the appropriate antibiotics in a 1 L Erlenmeyer flask. At saturation, the culture was diluted 50–fold into 5–7 L GMML medium in a fermentor flask with appropriate antibiotics. When $OD_{600\ nm}$ was 0.6–0.8, the air was replaced with $N_{2(g)}$. After 15 min, NH_2Y and DTT were added to final concentrations of 1 mM and 0.1 mM, respectively, and the growth continued as described above.

Several expression systems were tested: (1) the pBK–NH_2Y–RS/pBAD–α2 expression system was investigated, in which pBK–NH_2Y–RS carries the NH_2Y–RS gene under control of the constitutive *E. coli* Gln–RS promoter and terminator and a Kan^R marker, and vector pBAD–α2 carries the α2 gene with the appropriate amber codon under control of an L–Ara–inducible promoter and a *rrnB* terminator as well as the $mutRNA_{CUA}$ gene under control of a *lpp* promoter and *rrnC* terminator and a Tet^R marker; (2) The pBK–NH_2Y–RS/pMJ1–*nrdA* vector combination was examined, in which vector pMJ1–*nrdA* carries the *nrdA* gene with the amber codon under control of T7 promoter and terminator and a Amp^R marker. (3) The pAC–NH_2Y–RS/pMJ1–*nrdA* system was also examined, where pAC–NH_2Y–RS carries the NH_2Y–RS gene under control of *glnS′* promoter and *rrnB* terminator, six copies of the $mutRNA_{CUA}$ gene under control of a *proK* promoter and terminator and a Tet^R marker (Table 1).

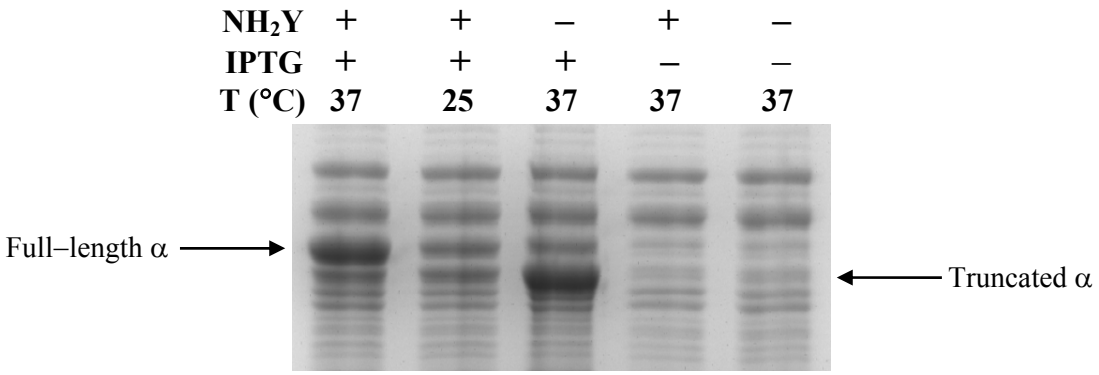

Figure S1: Expression of $Y_{730}NH_2Y$–α2. Cells were grown in the presence or absence of IPTG and NH_2Y/DTT and at 25° or 37 °C, as indicated, and the level of expression assessed by SDS PAGE. The position of protein bands for full–length α and truncated α are denoted by arrows.

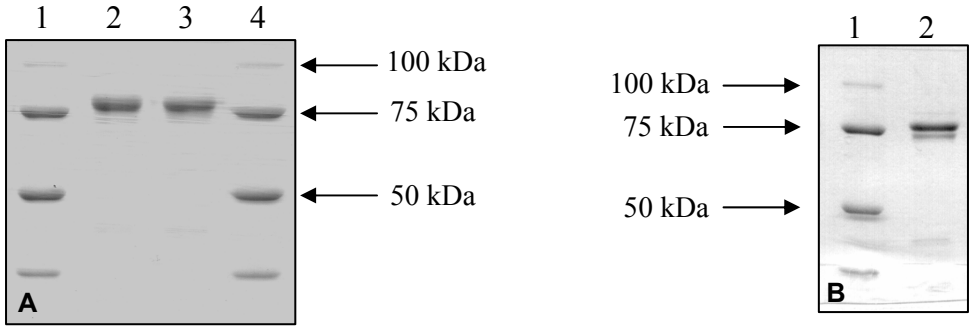

Figure S2: SDS PAGE analysis of purified $Y_{730}NH_2Y$–α2 (A) and $Y_{731}NH_2Y$–β2 (B). (A) Lanes (1) and (4), MW markers. The MW for each band is indicated. Lane (2), purified $Y_{730}NH_2Y$–α2 (1.5 μg). Lane (3), purified wt α2 (1.5 μg). (B) Lane (1), MW markers. The MW for each band is indicated. Lane (2), purified $Y_{731}NH_2Y$–α2 (1.5 μg).

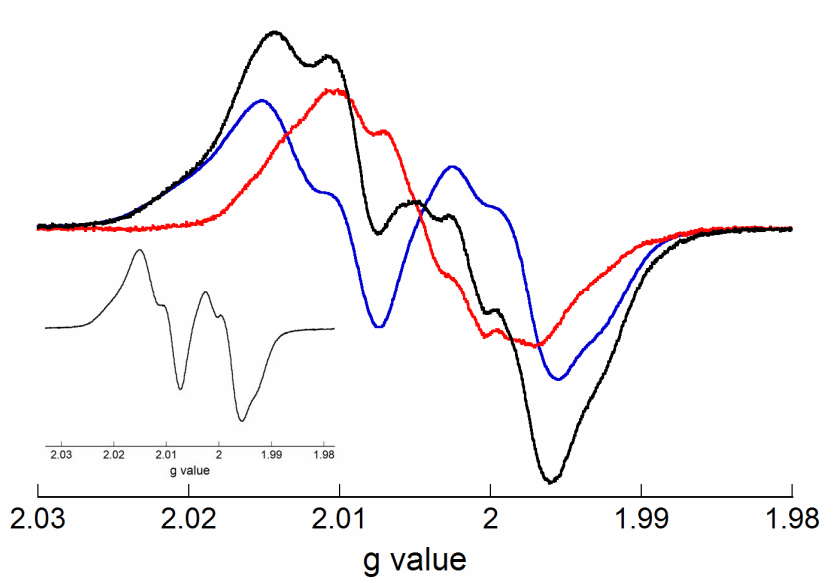

Figure S3: Reaction of $Y_{731}NH_2Y$–α2/ATP with wt β2/CDP monitored by EPR. The reaction components were mixed at 25°C to yield final concentrations of 20 μM $Y_{731}NH_2Y$–α2β2 complex, 1 mM CDP and 3 mM ATP. After 10 s, the reaction was quenched by hand–freezing in liquid N_2 and the EPR spectrum subsequently recorded at 77 K as described in the Methods section. Unreacted Y_{122}• (blue, 55 % of total spin), was subtracted to reveal the spectrum of NH_2Y_{730}• (red, 45 % of total spin). *Inset:* Reaction of $Y_{730}NH_2Y$–α2 with wt β2 in the absence of CDP/ATP.

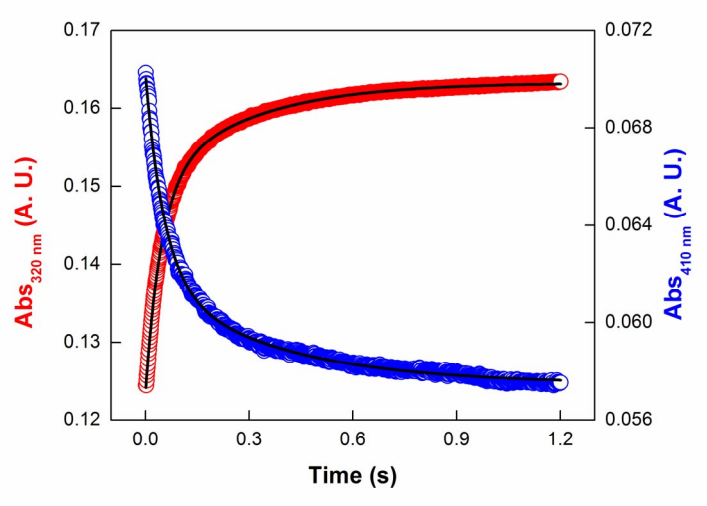

Figure S4: Kinetics of NH_2Y_{731}• formation. Pre–reduced $Y_{731}NH_2Y$–α2 (18 μM) and CDP (2 mM) in one syringe were mixed in a 1:1 ratio with β2 (18 μM) and ATP (3 mM) from another syringe. A total of 6 traces were averaged at 320 nm and 410 nm monitoring NH_2Y_{731}• formation (red) and Y_{122}• disappearance (blue), respectively. Black lines indicate bi–exponential fits to the data – see Table 2 for kinetic parameters.

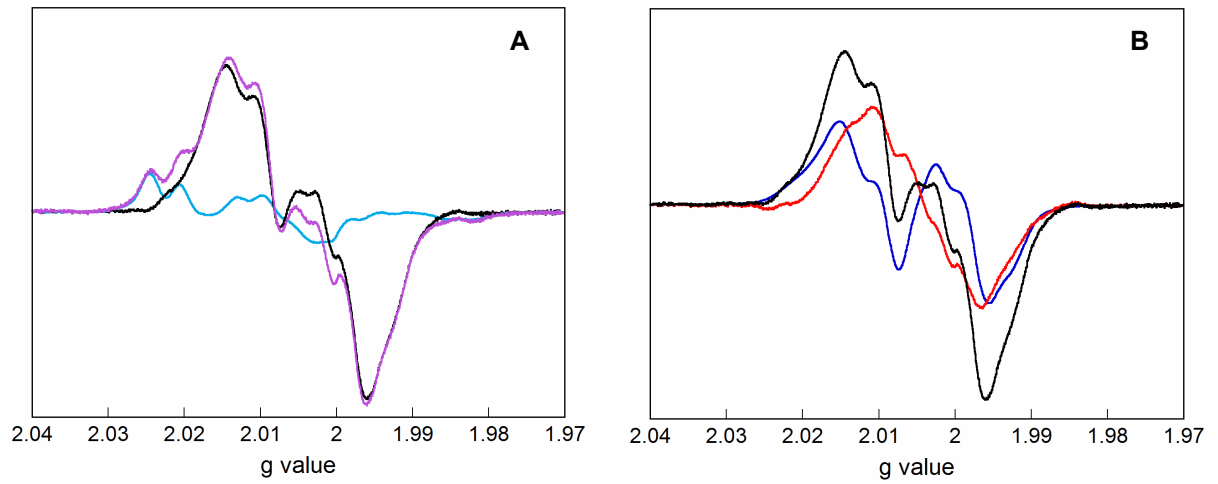

Figure S5: N_3ADP Assay for $Y_{731}NH_2Y$–α2. The reaction contained final concentrations of 20 μM $Y_{731}NH_2Y$–α2β2 (1.2 Y_{122}• / β2), 1 mM N_3ADP and 250 μM dGTP. After 20 s, it was freeze–quenched by hand in liquid N_2 and its EPR spectrum recorded. (A) Subtraction of N• (aqua, 20 % of total spin) from the observed spectrum (lavender trace) yields the black trace, which contains Y_{122}• and NH_2Y_{731}• signals. (B) Subtraction of Y_{122}• (blue, 41 % of total) from the resulting spectrum in (A) reveals the spectrum of NH_2Y_{731}• (red, 39 % of total spin). See Table 4 for quantitation of the concentration of each radical species.