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Replacement of Y₇₃₀ and Y₇₃₁ in the $\alpha 2$ Subunit of *Escherichia coli* Ribonucleotide Reductase with 3-Aminotyrosine using an Evolved Suppressor tRNA/tRNA-Synthetase Pair

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Abstract

Since the discovery of the essential tyrosyl radical (Y•) in *E. coli* ribonucleotide reductase (RNR), a number of enzymes involved in primary metabolism have been found that use transient or stable tyrosyl (Y) or tryptophanyl (W) radicals in catalysis. These enzymes engage in a myriad of charge transfer reactions that occur with exquisite control and specificity. The unavailability of natural amino acids that can perturb the reduction potential and/or protonation states of redox-active Y or W residues has limited the usefulness of site-directed mutagenesis methods to probe the attendant mechanism of charge transport at these residues. However, recent technologies designed to site-specifically incorporate unnatural amino acids into proteins have now made viable the study of these mechanisms. The class Ia RNR from *E. coli* serves as a paradigm for enzymes that use amino acid radicals in catalysis. It catalyzes the conversion of nucleotides to deoxynucleotides and utilizes both stable and transient protein radicals. This reaction requires radical transfer from a stable tyrosyl radical (Y₁₂₂•) in the β subunit to an active-site cysteine (C₄₃₉) in the α subunit, where nucleotide reduction occurs. The distance between the sites is proposed to be >35 Å. A pathway between these sites has been proposed in which transient aromatic amino acid radicals mediate radical transport. To examine the pathway for radical propagation as well as requirements for coupled electron and proton transfers, a suppressor tRNA/aminoacyl-tRNA synthetase (RS) pair has been evolved that allows for site-specific incorporation of 3-aminotyrosine (NH₂Y). NH₂Y was chosen because it is structurally similar to Y with a similar phenolic pK_a. However, at pH 7, it is more easily oxidized than Y by 190 mV (≈ 4.4 kcal/mol), thus allowing it to act as a radical trap. Here we present the detailed procedures involved in evolving an NH₂Y-specific RS, assessing its efficiency in NH₂Y insertion, generating RNR mutants with NH₂Y at selected sites, and determining the spectroscopic properties of NH₂Y• and the kinetics of its formation.

1. Introduction

Long-range electron transfer (ET) reactions are prevalent in biology, notably in photosynthesis and respiration, and in almost all cases occur between metal cofactors that are spaced 10 to 15 Å apart (Gray and Winkler, 1996; Marcus and Sutin, 1985; Moser *et al.*, 1992; Page *et al.*, 1999, 2003). ET theory, articulated by Marcus, has been immensely useful in dissecting the kinetics and thermodynamics of these reactions. More recently, however, the role of amino acid radicals as mediators of charge transport has been recognized (Cukier

and Nocera, 1998; Reece *et al.*, 2006). Oxidation of amino acids at neutral pH requires loss of an electron and a proton, implicating proton-coupled electron transfer (PCET).

Ribonucleotide reductases (RNRs), the topic of this chapter, serve as a paradigm for long-range PCET reactions, in which transient amino acid radicals are involved (Jordan and Reichard, 1998; Stubbe and Riggs-Gelasco, 1998; Stubbe and van der Donk, 1998; Stubbe *et al.*, 2003).

The *E. coli* RNR consists of two homodimeric protein subunits: $\alpha 2$ and $\beta 2$. The $\beta 2$ subunit contains a stable diferric tyrosyl radical ($Y_{122}^{\bullet}$) cofactor, which is essential for catalysis and is buried deeply within the protein (Ehrenberg and Reichard, 1972; Nordlund *et al.*, 1990; Reichard and Ehrenberg, 1983; Sjöberg *et al.*, 1978). The $\alpha 2$ subunit harbors the active site, where a cysteinyl radical ($C_{439}^{\bullet}$), generated by $Y_{122}^{\bullet}$ on radical transfer from $\beta 2$, initiates nucleotide reduction (Stubbe, 1990, 1998; Uhlin and Eklund, 1994). This intersubunit radical transfer reaction has been proposed to span a distance of $>35 \text{ \AA}$ on the basis of the Eklund docking model created from the structures of $\alpha 2$ and of $\beta 2$ (Eklund *et al.*, 2001; Uhlin and Eklund, 1994). A pathway of amino acids that gap the distance between the $Y_{122}^{\bullet}$ in $\beta 2$ and $C_{439}^{\bullet}$ in $\alpha 2$ has been proposed (Fig. 3.1) (Nordlund *et al.*, 1990; Stubbe *et al.*, 2003; Uhlin and Eklund, 1994). Several properties of the proposed radical transfer pathway in the *E. coli* class Ia RNR have made it difficult to examine with conventional methods. First, replacement of any residue involved in radical propagation by another natural amino acid via site-directed mutagenesis renders the enzyme inactive, thus precluding mechanistic studies (Climent *et al.*, 1992; Ekberg *et al.*, 1996; Stubbe *et al.*, 2003). Second, pre-steady-state kinetic experiments have revealed that radical transfer is preceded by a slow conformational change (Ge *et al.*, 2003). Consequently, to study this process, the rate-limiting step must be changed from the physical step to the radical transfer step. Finally, the system has evolved to avoid buildup of any intermediates during this process, which could result in loss of the essential radical (Licht and Stubbe, 1999). In *E. coli* RNR, the $Y_{122}^{\bullet}$ has a lifetime of several days (Atkin *et al.*, 1973).

Site-specific replacement of the tyrosines within the radical transfer pathway (Fig. 3.1) with appropriately designed unnatural amino acids could potentially allow for investigation of the length of the pathway and the existence of transient radical intermediates. Therefore, we have sought modified tyrosines that can block radical transfer because of their inability to be oxidized to the corresponding radical form, report on radical transfer because of their ease of oxidation relative to tyrosine, or change the rate-limiting step from the conformational change to radical transfer by modulating the radical reduction potential over several hundred mV relative to tyrosine in the physiological pH range.

To incorporate a thermodynamic block into the radical transfer pathway, we previously chose 3-nitrotyrosine (NO_2Y) as an analogue because it is substantially more difficult to oxidize than Y (by 200 mV at pH 7) (Yee *et al.*, 2003b). This block in the pathway could potentially allow accumulation of radical intermediates preceding the site of incorporation. NO_2Y also functioned as a reporter on the ability of the protein environment to perturb the phenolic pK_a , essential in understanding PCET mechanisms. To insert a radical trap into the pathway, we chose 3,4-dihydroxyphenylalanine (DOPA) because it is substantially easier to oxidize than Y (by 270 mV at pH 7) (Seyedsayamdost and Stubbe, 2006). This

thermodynamic trap then served as a readout for the involvement of residue 356 in radical transfer. Finally, we chose a series of fluorotyrosine analogues (F_nYs , $n = 1-4$) which perturb the phenolic pK_a and modulate the radical reduction potential relative to Y by -40 to $+270$ mV, depending on the reaction pH and the number of fluorines (Seyedsayamdost *et al.*, 2006a). These analogues allowed us to change the rate-limiting step in RNR and to uncouple the electron and proton flows at a given residue (Seyedsayamdost *et al.*, 2006b; Yee *et al.*, 2003a). We recently reported detailed procedures for replacement of Y_{356} in $\beta 2$ by expressed protein ligation (EPL) methods (Muralidharan and Muir, 2006; Pellois and Muir, 2006) to site-specifically insert NO_2Y , DOPA, and F_nYs (Seyedsayamdost *et al.*, 2007c).

To study residues involved in radical transfer in $\alpha 2$ in a similar fashion, we have considered two methods for the insertion of unnatural amino acids. The first method, semisynthesis of the α subunit of RNR using EPL, is not feasible because of the large size of $\alpha 2$ (each monomer is 761 amino acids), its poor solubility, and the position of the residues to be replaced (Y_{730}/Y_{731} , Fig. 3.1). We have therefore employed an alternative method for site-specific insertion of unnatural amino acids. Herein, we detail our approach for inserting the unnatural tyrosine analogue 3-aminotyrosine (NH_2Y) into the $\alpha 2$ subunit of RNR using suppressor tRNA/aminoacyl-tRNA synthetase (RS) methodologies (Seyedsayamdost *et al.*, 2007b; Wang and Schultz, 2004; Xie and Schultz, 2005).

2. Site-Specific Insertion of Unnatural Amino Acids using the Suppressor tRNA/RS Method

Schultz and colleagues have developed robust methods for site-specific incorporation of a wide range of unnatural tyrosine and phenylalanine analogues, *in vivo* (Wang and Schultz, 2004; Xie and Schultz, 2005, 2006). In collaboration with the Schultz lab, we have been able to apply this methodology to incorporate NH_2Y into α (Seyedsayamdost *et al.*, 2007a,b). In this method, six residues at or near the active site of the *Methanococcus jannaschii* Tyr-RS were randomized to generate a library of RSs. The resulting library was then subjected to a set of positive and negative selections (see section 4) to find one or several RSs that can selectively charge the cognate *M. jannaschii* tRNA_{Tyr} with NH_2Y and not with Y. This suppressor tRNA, designated mutRNA_{CUA}, contains an amber stop codon and was optimized for interacting with the library of RSs (Wang and Schultz, 2001). A number of criteria must be satisfied by the selected *M. jannaschii* mutRNA_{CUA}/RS pair to make it suitable in this procedure (Xie and Schultz, 2005). First, the mutRNA_{CUA}/RS pair must be orthogonal to the expression host's tRNA/RS pairs; that is, the amber suppressor mutRNA_{CUA} must not be charged by any RSs from the host organism *in vivo*. Second, the selected RS must charge only its cognate mutRNA_{CUA} and not any of the host's tRNAs. Third, *in vivo* RSs must not be able to charge their cognate tRNAs with the unnatural amino acid. Fourth, the unnatural amino acid must be taken up by the host cell and must not be toxic. Finally, the gene of interest, with the amber stop codon at the residue where the unnatural amino acid is to be inserted, must be expressed inside the host. If all of these criteria are met, then the gene of interest and the genes for the evolved mutRNA_{CUA}/RS pair specific for the unnatural amino acid are transformed into the host, and the expression of the

protein of interest results in the incorporation of the unnatural amino acid with high fidelity and specificity.

Both the expressed protein ligation method (Dawson and Kent, 2000; Gariat and Muir, 2003; Mootz *et al.*, 2003; Muir, 2003; Perler, 2005) and the suppressor tRNA/RS method (Wang and Schultz, 2004; Wang *et al.*, 2006; Zhang *et al.*, 2003) have been used *in vitro* and *in vivo*, each with its advantages and disadvantages. While we have used EPL extensively with the β subunit and gained important insights into radical transfer, the tRNA/RS method promises to be more robust for incorporation of unnatural amino acids into both RNR subunits for several reasons. First, it allows incorporation of the unnatural amino acid anywhere in the protein, not just at the C- or N-terminus. Muir and colleagues have developed methods for insertion of probes in the middle of proteins (Cotton *et al.*, 1999); however, refolding of α or β is not an option, and therefore incorporation of unnatural amino acids by these techniques into the center of the protein would not be viable. Second, the β subunit of RNR is a dimer. Therefore, the semisynthesis of β_2 by EPL required separation of the full-length dimeric β_2 , the heterodimeric $\beta\beta'$ (composed of a full-length monomer, β , and a truncated monomer, β') and the doubly truncated dimer β'_2 . While this separation was possible, it came at the expense of protein yield. Third, expressed protein ligation of β_2 required two additional mutations to provide sufficient quantities of semisynthetic β_2 for physical biochemical methods necessary to study radical transfer in RNR. A serine residue (S₃₅₄) needed to be changed to a cysteine for the ligation reaction, and the residue at the site of the activated thioester-intein construct (V₃₅₃) needed to be replaced with a less bulky amino acid (Gly) to increase the yield of the ligated product (Yee *et al.*, 2003b). With the tRNA/RS method, insertions can be made with a single unnatural point mutation. In addition, the requirement to assemble the essential diferric Y₁₂₂• cofactor of β_2 and the sensitivity of the unnatural amino acids to oxidation made the intein method challenging at every step.

As with every method, there are a number of problems that may be encountered with the suppressor tRNA/RS method. The unnatural amino acid of interest might not be readily taken up into the host cell or may be toxic. In addition, each unnatural amino acid requires evolution of a new RS. As documented subsequently, the suppressor tRNA/RS pair has been highly successful in site-specific insertion of NH₂Y into RNR and has allowed us to perform mechanistic studies, which would not have been accessible by other methods.

3. NH₂Y, a Y Analogue for Investigating Enzymatic PCET Reactions

3.1. Overview: Choice of NH₂Y

NH₂Y was chosen as a probe because its radical reduction potential of 0.64 V (pH 7.0) is 0.19 V lower than that of Y (DeFelippis *et al.*, 1991), which suggests that it might act as a radical trap and directly report on the participation of residues Y₇₃₀ and Y₇₃₁ in radical transfer (Fig. 3.1). In addition, the ease of synthesis of NH₂Y (Seagle and Cowgill, 1976) and its kinetic stability to oxidation relative to other low reduction potential radical traps, such as DOPA (Jovanovic, 1994), make it a more practical target. The characterization of the UV-vis and EPR spectroscopic properties of the oxidized NH₂Y, the amino tyrosyl radical (NH₂Y•), will allow NH₂Y to serve as a probe for enzymes that are thought to

employ transient Y•s, or modified Y•s in catalysis or in electron transfer (Seyedsayamdost *et al.*, 2007b). The ability to incorporate NH₂Y may also be of more general use for site-specific appendage of probes, as recent studies have shown that NH₂Y itself can be derivatized with fluorescent dyes (Hooker *et al.*, 2004; Kovacs *et al.*, 2007). Further, we have shown that NH₂Y can be used as a redox-active distance probe to determine molecular distances of 15 to 80 Å between two paramagnetic sites within an enzyme or enzyme complex (Bennati *et al.*, 2003; Seyedsayamdost *et al.*, 2007a).

Before performing directed-evolution on the library of RSs to select for an NH₂Y-RS, three criteria must be fulfilled by NH₂Y: it must be taken up by *E. coli* cells, it must not be toxic, and it must not be incorporated into proteins by any endogenous RSs. In this section, we outline the procedures used to determine the uptake and toxicity of NH₂Y.

3.2. Protocol for assessing uptake and toxicity of NH₂Y in *E. coli*

To assess the uptake of NH₂Y by *E. coli* (Xie and Schultz, 2005), growth of DH10B cells (Invitrogen) was initiated in the absence of antibiotics in two 50-mL Erlenmeyer flasks containing 15 mL of glycerol minimal media + leucine (GMML), which contains final concentrations of 1% (v/v) glycerol, 1× M9 salts (Sigma-Aldrich), 0.05% (w/v) NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, and 0.3 mM L-leucine (Sigma-Aldrich). The inclusion of L-leucine is important, as it greatly enhances growth rates. One flask was supplemented with 1 mM NH₂Y (Sigma-Aldrich) and 0.1 mM DTT (Mallinckrodt), while the other served as a control. The cultures were grown to saturation, harvested by centrifugation (6000g, 8 min) and each supernatant was transferred to a Falcon tube. The cell pellets were washed twice by resuspension in 1 mL of GMML, the cells isolated by centrifugation (6000g, 8 min), and the supernatant discarded. The washed cell pellets were then resuspended in 0.2 mL of BugBuster Protein Extraction Reagent (Novagen) and shaken at 37 °C for 1 h. Cell debris was pelleted by centrifugation (20,000g, 15 min) and the crude extract filtered using Microcon YM-10 membranes (Millipore). The filtered crude extract was subjected to LC-ESI-MS analysis using a Zorbax SB-C18 column (5 μm, 4.6 × 150 mm, Agilent Technologies) with a linear gradient from 5 to 25% MeCN in 0.1% TFA solution over 8 min at a flow rate of 0.5 mL/min. Under these conditions, NH₂Y elutes at ≈13% MeCN. ESI-MS sampling of the eluate was performed under positive ionization mode. Authentic NH₂Y standard solutions were prepared in water and chromatographed under identical conditions. NH₂Y from the crude extract was identified on the basis of comparison with the retention time of authentic NH₂Y, as well as by ESI-MS results.

3.3. Results

The DH10B *E. coli* cells grew normally in the presence of NH₂Y, indicating that it is not toxic, though the presence of DTT, included in the medium to maintain a reducing environment, reduced the growth rate by 25 to 35% (data not shown). Previous studies with DOPA had indicated that inclusion of DTT in the growth medium significantly increased the lifetime of reduced DOPA (Alfonta *et al.*, 2003). Therefore, DTT was maintained in all our experiments despite the reduction in the growth rate.

The HPLC of crude extracts clearly identified a peak corresponding to NH_2Y , as determined by the retention time and the ESI-MS results, which were identical to those of the authentic standard NH_2Y solution ($[\text{M}+\text{H}]^+_{\text{exp}}$ 197, $[\text{M}+\text{H}]^+_{\text{obs}}$ 196.9). The uptake was not determined as a function of time but rather at culture saturation. We were also able to detect NH_2Y in the supernatant after the cells were harvested by centrifugation, which showed that NH_2Y remains reduced in the culture throughout the time course of the growth. Together, the results showed that NH_2Y is not toxic and is taken up into *E. coli*.

The structure of *E. coli* Tyr-RS was examined to evaluate whether it would be able to bind NH_2Y and consequently charge tRNA_{Tyr} with this analogue. Previous reports had demonstrated that *E. coli* Tyr-RS is unable to adenylate 3-iodo-Tyr (Kiga *et al.*, 2002), suggesting that NH_2Y , which is similar in size, will unlikely be a substrate for Tyr-RS. In support of this hypothesis, the structures of the adenylation domain of *E. coli* Tyr-RS in the presence of Tyr and adenylated Tyr (Kobayashi *et al.*, 2005) show that residues Y₃₇, Q₁₇₉ and Q₁₉₅, and D₁₈₂ are within 3.4 Å of the phenol C₃ and phenol C₅ carbon atoms, respectively, which are ortho to the hydroxyl group at C₄. This analysis suggests that NH_2Y would be unable to bind *E. coli* Tyr-RS because of unfavorable steric interactions with its 3-amino moiety. Although this conclusion needs to be further verified experimentally by isolating and assaying the *E. coli* Tyr-RS, the foregoing arguments suggest that NH_2Y meets the final criterion for commencing directed evolution of a specific NH_2Y -RS.

4. Directed Evolution of NH_2Y -RS in *E. coli*

In this section, we summarize the general selection process used to isolate NH_2Y -RS. The protocols used to select for an NH_2Y -RS are similar to those previously described in detail and are summarized in Fig. 3.2 (Wang and Schultz, 2004; Xie and Schultz, 2005). The positive selection is performed in DH10B *E. coli* cells containing the positive selection plasmid, pREP/YC-J17 (Santoro *et al.*, 2002), which carries a tetracycline marker (Tet^R), and plasmid pBK-JYRS (Wang *et al.*, 2001), which contains the library of RSs and a kanamycin marker (Kn^R). The positive selection is based on suppression of an amber stop codon (TAG) at a permissive site in the chloramphenicol acetyl transferase (CAT) gene when DH10B *E. coli* cells are grown in the presence of chloramphenicol (Cm), Kn, Tet, NH_2Y /DTT, and the cognate tRNA on GMML plates. Surviving clones carry RSs that are functional with the host cell's translation machinery and incorporate NH_2Y or natural amino acids into CAT in response to the amber stop codon. This leads to production of CAT and survival of the clones in the presence of 60 $\mu\text{g}/\text{mL}$ Cm. In addition, the positive selection plasmid contains a GFPuv gene under the control of the T7 promoter and a T7 RNA polymerase, which contains amber codons at two permissive sites. Suppression of the amber stop codons generates T7 RNA polymerase, which drives the expression of GFPuv resulting in green fluorescence in the desired clones. Therefore, Cm resistance and green fluorescence may be monitored to isolate clones that carry the desired RSs (Xie and Schultz, 2005).

After the positive selection, the cells are scraped from the plates and isolated by centrifugation. The vectors pREP/YC-J17 (≈ 10 kb) and pBK-JYRS (≈ 3 kb) are isolated using the Qiagen Miniprep Kit and separated on a 1% agarose gel. Then, pBK-JYRS is extracted using the Qiagen Gel Extraction Kit and transformed into DH10B *E. coli* cells

carrying the negative selection plasmid, pLWJ17B3 (Chin *et al.*, 2002; Wang *et al.*, 2003). The negative selection is based on lack of suppression of three amber codons in the barnase gene, which codes for a ribonuclease, when the cells are grown in the absence of NH₂Y in LB. The RSs that can charge the suppressor mutRNA_{CUA} with a natural amino acid generate barnase, which kills the cells. Thus, this selection removes RSs that are not specific for NH₂Y. The surviving clones carry RSs that do not incorporate any natural amino acids in response to the amber stop codon. The RSs are then examined to determine the fidelity and specificity of NH₂Y insertion in a model system, as described in the subsequent section.

Four rounds of positive and three rounds of negative selections were required to obtain RSs that were specific for NH₂Y and did not cross-react with natural amino acids (Seyedsayamdost *et al.*, 2007b). The RS that conferred the highest degree of Cm resistance ($\approx 110 \mu\text{g/mL}$) was selected and amplified. The plasmid carrying this RS was designated pBK-NH₂Y-RS (Seyedsayamdost *et al.*, 2007b). While structural studies have not yet been performed on this RS, DNA sequencing revealed a Q₃₂ instead of the wild-type Y₃₂ (note that this Y corresponds to Y₃₇ in the *E. coli* Tyr-RS discussed earlier). In the wild-type *M. jannaschii* RS, the phenol oxygen of Y₃₂ is positioned within 3.4 Å of the phenolic C-atom ortho to the hydroxyl group of the Tyr ligand (Kobayashi *et al.*, 2003; Turner *et al.*, 2006; Zhang *et al.*, 2005). In the selected NH₂Y-RS, this residue is Q, which indicates that the binding pocket has been expanded to allow accommodation for, and perhaps favorable H-bonding interactions with, the *o*-NH₂ moiety of NH₂Y.

5. Examination of the Fidelity and Specificity of NH₂Y Incorporation in a Protein Expressed in *E. coli*

5.1. Overview: The Z-domain as a model

A variety of expression systems for small proteins have been developed by the Schultz lab to assess the fidelity and specificity of unnatural amino acid incorporation. In our case, the efficiency of NH₂Y incorporation using pBK-NH₂Y-RS was tested using the C-terminally His-tagged Z-domain of protein A (the Z-domain) (Nilsson *et al.*, 1987; Wang *et al.*, 2003; Zhang *et al.*, 2002). There are several advantages and disadvantages to using small model proteins such as the Z-domain. The advantages are that the Z-domain is small and stable, allowing for robust expression and rapid purification at room temperature. More important, the incorporation of unnatural Y analogues can be assessed using simple analytical methods, such as MALDI-TOF-MS. Even small modifications, such as addition of an NH₂ group ($MW = 15 \text{ Da}$) can be easily detected using this method. Further, trypsin digests result in a small set of peptides that can be sequenced using MS methods. In contrast, the Z-domain is a poor surrogate for target enzymes, which are typically larger, less robust, and/or multimeric in nature. Therefore, incorporation of an unnatural amino acid into the Z-domain does not guarantee that it will also occur in the target protein, and thus expression of the latter must be optimized. In addition, the Z-domain is subject to multiple forms of posttranslational modification that complicates MS analysis (Wang *et al.*, 2003). Nevertheless, it provides a rapid test for the efficiency of the evolved RS.

5.2. Protocol for incorporation of NH₂Y into the Z-domain

The vectors used to incorporate NH₂Y into the Z-domain were pBK-NH₂Y-RS and pLEIZ (Wang *et al.*, 2003; Zhang *et al.*, 2002). The latter contains the Z-domain gene with an amber stop codon at residue 7 and is under the control of a T7 promoter. It also contains the cognate mutRNA_{CUA} and a Cm^R marker. The plasmids were cotransformed into BL21(DE3)-competent cells (Invitrogen) using the manufacturer's instructions and plated on LB/agar plates containing 50 µg/mL Kn and 35 µg/mL Cm. All subsequent cultures were grown in the presence of Kn (50 µg/mL) and Cm (35 µg/mL) at 37 °C. A single colony from the plate was inoculated into 5 mL of 2YT medium (Becton Dickinson) and grown to saturation (≈13 h). One mL of this saturated culture was diluted into 25 mL 2YT medium and grown to saturation overnight (≈11 h). Ten mL of that culture was then diluted into each of 2 × 1 L Erlenmeyer flasks containing 250 mL of GMML. When the OD_{600 nm} reached 0.65 (9 h), one of the cultures was supplemented with final concentrations of 1 mM NH₂Y and 0.1 mM DTT. The other culture was supplemented only with DTT (0.1 mM). Fifteen minutes after addition of NH₂Y/DTT (or DTT), IPTG (Sigma-Aldrich) was added to each culture to a final concentration of 1 mM. After 5 h, cells were harvested by centrifugation. The Z-domain produced from these cultures grown in the presence and absence of NH₂Y was then purified by Ni²⁺ affinity chromatography (Novagen) under denaturing conditions, per manufacturer's instructions. After purification, the Z-domain was analyzed by SDS-PAGE and MALDI-TOF MS. For MALDI-TOF MS, the Z-domain was exchanged into water by dialysis at 4 °C using 1-kDa molecular weight cutoff membranes (SpectroPor), and mass spectra were subsequently obtained under positive ionization mode at the Scripps Center for Mass Spectrometry.

5.3. Results

The SDS-PAGE analysis of the expression and purification of Z-domain in the presence and absence of NH₂Y is shown in Fig. 3.3A. The results show that expression of the Z-domain in the presence of NH₂Y/DTT in GMML resulted in suppression of the amber stop codon. Typically, 5 mg of Z-domain per liter of culture were obtained after purification. MALDI-TOF MS analysis of the sample yielded four major peaks (Fig. 3.3B) with MW = 7812, 7854, 7943, and 7985 Da, corresponding to K₇NH₂Y-Z-domain minus the first Met (MW_{exp} = 7813 Da), its acetylated form (MW_{exp} = 7855 Da), full-length K₇NH₂Y-Z-domain (MW_{exp} = 7944 Da), and its acetylated form (MW_{exp} = 7986 Da), respectively. As discussed earlier, the Z-domain is subject to posttranslational modifications that result in acetylation of its N-terminus and removal of its first Met residue (Wang *et al.*, 2003). Importantly, K₇Y-Z-domain, which would result from mischarging of mutRNA_{CUA} with Y in place of NH₂Y, was not detected in the MALDI-TOF mass spectra. These results indicate that NH₂Y-RS is efficient and specific at suppressing the amber stop codon and inserting NH₂Y. In support of this finding, in the absence of NH₂Y, the amount of Z-domain produced was less than the lower limit of detection (≈0.5 mg/L of culture). Therefore, in the absence of NH₂Y, the amber stop codon is not suppressed, resulting in the lack of expression or low levels of expression of the Z-domain.

6. Generation of Y₇₃₀NH₂Y- α 2 and Y₇₃₁NH₂Y- α 2

6.1. Overview

Having selected an NH₂Y-RS and demonstrated its ability to efficiently incorporate NH₂Y into the Z-domain, we next attempted to insert NH₂Y into the α subunit of RNR. As discussed previously, while the Z-domain allows for rapid assessment of the quality of the evolved RS, the conditions used for optimized expression and suppression of the amber stop codon in the target protein are usually different, as we and other research groups have experienced (Farrell *et al.*, 2005; Neumann *et al.*, 2008). In this section, we describe our initial unsuccessful attempts at incorporating NH₂Y into α 2, as well as our detailed protocol for successful production of NH₂Y- α 2s in 100-mg quantities with high specificity.

6.2. Unsuccessful attempts to incorporate NH₂Y into α 2

Successful incorporation of NH₂Y into α 2 first requires identification of a condition that yields high levels of expression of α 2, and then suppression of the amber stop codon under the same growth condition. We first attempted to insert NH₂Y into α 2 using the procedure successful with the Z-domain. pBAD-*nrdA* (or *nrdA* with a site-specifically encoded amber codon) carries the gene for α 2 under the control of an L-Ara-inducible promoter with an *rrnB* terminator and the mutRNA_{CUA} gene under the control of a *lpp* promoter with an *rrnC* terminator and a Tet^R marker (Seyedsayamdost *et al.*, 2007b; Zhang *et al.*, 2003). pBK-NH₂Y-RS contains the gene for NH₂Y-RS described previously under the control of the constitutive *E. coli* Gln-RS promoter. Also, pBK-NH₂Y-RS and pBAD-*nrdA* contain compatible origins of replication, ColE1 and p15A, respectively.

Initially, we examined pBAD-*nrdA* alone to evaluate expression levels of α 2 and α 2 containing an amber stop codon (at residue 730) in GMML. In the former case, α 2 was overexpressed to $\approx$ 10% of total protein; in the latter case, truncated α 2 was overexpressed at similar levels. Ensuring expression in the absence of pBK-NH₂Y-RS is essential; however, it does not guarantee successful suppression of the amber stop codon.

Next, we examined the expression levels and amber stop codon suppression in cells cotransformed with pBAD-*nrdA* and pBK-NH₂Y-RS, grown in NH₂Y/DTT-supplemented GMML. Different conditions of induction and timing of addition of NH₂Y relative to induction were investigated. In all cases, levels of expression of α 2 were low, as determined by SDS-PAGE. Western blot analysis indicated that the levels of α 2 in the presence of NH₂Y were $\approx$ 2-fold that of endogenous α 2 (α 2 is an essential gene in *E. coli*, data not shown). We attempted growth under anaerobic conditions or aerobic growth in the presence of hydroxyurea. In the former case, the anaerobic class III RNR is operative in *E. coli*; therefore, wild-type class I β 2 is not expressed. In the latter case, addition of hydroxyurea leads to reduction of the essential Y₁₂₂• in class I β 2 so that it cannot react with NH₂Y- α 2, if it is in fact expressed. In both cases, the reaction of wild-type β 2 with NH₂Y- α 2, which could lead to trapping of an NH₂Y• and subsequent inactivation of RNR, would be minimized. However, neither of the modifications in growth conditions resulted in significant overexpression of α 2.

Continuous technological refinements from the Schultz lab have indicated that mutRNA_{CUA} is often the limiting factor in production of sufficient levels of a recombinant target protein containing an unnatural amino acid (Ryu and Schultz, 2006). Failure to overexpress NH₂Y- α 2, as described previously, might therefore have been due to limiting amounts of mutRNA_{CUA} inside the cell. To increase the amounts of mutRNA_{CUA}, we next attempted expression of α 2 with pAC-NH₂Y-RS, a vector developed by the Schultz lab to overcome this problem. pAC-NH₂Y-RS has six genetic copies of mutRNA_{CUA} under the control of a *proK* promoter and terminator (Ryu and Schultz, 2006; Seyedsayamdost *et al.*, 2007b). It also carries the NH₂Y-RS gene under the control of a *glnS'* promoter, an *rrnB* terminator, and a Tet^R marker. While our analysis to understand the low levels of overexpression of NH₂Y- α 2 was in progress, we found, as described subsequently, that pTrc-*nrdA* and pAC-NH₂Y-RS allowed overexpression of α 2 and incorporation of NH₂Y; thus, this vector combination became the focus of our efforts. We have not yet reexamined expression NH₂Y- α 2 with pBAD-*nrdA* and pAC-NH₂Y-RS and do not entirely understand the basis for the differences in the levels of expression of α 2 with different vector combinations.

6.3. Successful incorporation of NH₂Y into α 2

Reports of successful overexpression of *E. coli* nitroreductase using pTrc for production of the target protein (Jackson *et al.*, 2006) prompted us to investigate the pTrc-*nrdA*/pAC-NH₂Y-RS vector combination, simultaneously with efforts described in the preceding section. Thus, *nrdA* was cloned into the pTrc vector to generate pTrc-*nrdA*. This vector contains *nrdA* with an amber stop codon under the control of the highly active trp/lac (*trc*) promoter and an Amp^R marker (Amann *et al.*, 1988). Its p15a replicon is compatible with the ColE1 origin of pAC-NH₂Y-RS. Expression of α 2 from pTrc-*nrdA* in DH10B cells was examined first. Expression and purification yielded 10 mg α 2 per gram of wet cell paste, a 2 $\times$ to 4 $\times$ greater yield than with vector pMJ1-*nrdA*, which we have routinely used to generate wild-type and mutant α 2s (data not shown) (Mao *et al.*, 1989; Seyedsayamdost *et al.*, 2007b). The activity of α 2 produced from pTrc-*nrdA* was similar to that produced from pMJ1-*nrdA* ($\approx$ 2500 nmol/min mg by the spectrophotometric assay) (Ge *et al.*, 2003). Therefore, pTrc-*nrdA* was suitable for production of α 2, allowing expression of NH₂Y- α 2 from pTrc-*nrdA* and pAC-NH₂Y-RS.

6.4. Protocol for successful expression of NH₂Y- α 2s

Vectors pTrc-*nrdA*₇₃₀TAG and pTrc-*nrdA*₇₃₁TAG were generated from pTrc-*nrdA* using the site-directed mutagenesis kit (Stratagene), as already described (Seyedsayamdost *et al.*, 2007b). *E. coli* DH10B cells were transformed with pTrc-*nrdA*₇₃₀TAG and pAC-NH₂Y-RS and grown at 37 °C on LB/agar plates containing Amp (100 μ g/mL) and Tet (25 μ g/mL) for two days. All liquid culture growths contained Amp (100 μ g/mL) and Tet (25 μ g/mL) and were carried out in a shaker/incubator at 37 °C and 200 rpm. A single colony from the plate was inoculated into 5 mL of 2YT medium and grown to saturation ($\approx$ 2 days). The 5 mL saturated culture was diluted into 180 mL of 2YT medium and grown to saturation ($\approx$ 1 day). Twenty five mL of this culture were then inoculated into each of 6 $\times$ 6 L Erlenmeyer flasks, each containing 1 L of GMM medium supplemented with D-biotin (1 μ g/mL, Sigma-Aldrich), thiamine (1 μ g/mL, Sigma-Aldrich) and a 1 $\times$ heavy metal stock solution. A 1000 $\times$ heavy metal stock solution contains the following per liter, as previously described

(Farrell *et al.*, 2005): 500 mg of $\text{MoNa}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$, 250 mg of CoCl_2 , 175 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 8.75 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.25 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.25 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1 g of H_3BO_3 in 1 M HCl. When $\text{OD}_{600 \text{ nm}}$ reached 0.6 (12 to 18 h), NH_2Y and DTT were added to final concentrations of 1 mM and 0.1 mM, respectively. After 15 min, IPTG was added to a final concentration of 1 mM and the growth continued for 4.5 h, at which point the cells were harvested by centrifugation, frozen in liquid N_2 and stored at -80°C . Expression of $\text{Y}_{731}\text{NH}_2\text{Y}-\alpha 2$ was carried out in identical fashion using pTrc-*nrdA*₇₃₁TAG and pAC- NH_2Y -RS.

6.5. Results

Using the foregoing expression procedure, typically 1.5 g of wet cell paste was obtained per liter of culture. The results for the expression of $\text{Y}_{731}\text{NH}_2\text{Y}-\alpha 2$ in DH10B cells doubly transformed with pAC- NH_2Y -RS and pTrc-*nrdA*₇₃₁TAG are shown in Fig. 3.4A. In the presence of the IPTG inducer and NH_2Y /DTT, the amber stop codon is suppressed and $\text{NH}_2\text{Y}-\alpha 2$ is overexpressed. In the absence of NH_2Y , overproduction of only truncated $\alpha 2$ is observed. Finally, in the absence of inducer IPTG and NH_2Y , no expression of $\alpha 2$ occurs. A similar profile was obtained for the expression of $\text{Y}_{730}\text{NH}_2\text{Y}-\alpha 2$ (Fig. 3.4B).

6.6. Protocol for purification of $\text{NH}_2\text{Y}-\alpha 2$ s

All purification steps were carried out at 4°C . Typically, 10 g of wet cell paste (from ≈ 6 L of growth) were used for purification. Each gram of cell paste was resuspended in 5 mL of $\alpha 2$ buffer, which consists of 50 mM Tris, 1 mM EDTA, pH 7.6, supplemented with 1 mM PMSF (Sigma-Aldrich) and 5 mM DTT. PMSF and DTT were added from a 100 mM stock in isopropanol and a 1 M stock in water, respectively. The cells were lysed using a single passage through a French pressure cell operating at 16,000 psi. After lysis, cell debris was removed by centrifugation (15,000g, 35 min, 4°C). The crude extract was transferred to a 100-mL beaker. DNA was precipitated by dropwise addition of 0.2 vol of $\alpha 2$ buffer containing 1 mM PMSF, 5 mM DTT, and 8 % (w/v) streptomycin sulfate (Sigma-Aldrich). The mixture was stirred for an additional 15 min, and the precipitated DNA was removed by centrifugation (15,000g, 35 min, 4°C). The supernatant was transferred to a 100-mL beaker and 3.9 g of solid $(\text{NH}_4)_2\text{SO}_4$ were added per 10 mL of supernatant over 15 min, which corresponds to 66% $(\text{NH}_4)_2\text{SO}_4$ saturation. The mixture was stirred for an additional 30 min and the precipitated protein was isolated by centrifugation (15,000g, 45 min, 4°C). The supernatant was discarded and the pellet redissolved in 3 to 4 mL of $\alpha 2$ buffer containing 1 mM PMSF and 5 mM DTT. This solution was desalted on a Sephadex G-25 column (1.5 cm $\times$ 25 cm, 45 mL, Sigma-Aldrich), which had been equilibrated in $\alpha 2$ buffer. The desalted protein was loaded directly onto a dATP affinity column (1.5 cm $\times$ 4 cm, 6 mL), which had been equilibrated in $\alpha 2$ buffer, at 0.5 mL/min (Berglund and Eckstein, 1972, 1974). The column was washed with 10 column-volumes of $\alpha 2$ buffer containing 1 mM PMSF and 5 mM DTT, followed by 2 volumes of $\alpha 2$ buffer containing 5 mM DTT. Then, $\text{NH}_2\text{Y}-\alpha 2$ was eluted in 3 to 4 column-volumes of $\alpha 2$ buffer containing 15 mM MgSO_4 , 10 mM ATP (Sigma-Aldrich), and 10 mM DTT. Subsequently, ATP was removed using a Sephadex G-25 column as described earlier and $\text{NH}_2\text{Y}-\alpha 2$ concentrated to $\approx 40 \mu\text{M}$ using a Centriprep concentration device (Millipore). Concentrated $\text{NH}_2\text{Y}-\alpha 2$ was divided into 0.5- to 1-mL

aliquots, flash frozen, and stored at -80°C . Typically, 4 to 6 mg of pure $\text{NH}_2\text{Y-}\alpha 2$ were obtained per gram of wet cell paste.

6.7. Assessment of the fidelity and specificity of NH_2Y insertion into $\alpha 2$

The analytical methods available for assessing the level of NH_2Y incorporation into the Z-domain are not feasible with $\alpha 2$. For example, incorporation of NH_2Y results in a 0.017% mass increase ($\alpha_{\text{MW}} \approx 86 \text{ kDa}$) in $\alpha 2$, as a result of the *o*- NH_2 moiety, which cannot be measured by ESI-MS methods. Further, analysis of trypsin digestion products of $\alpha 2$ is complicated given the large number of resulting peptides. Therefore, assessment of the efficiency of NH_2Y incorporation into $\alpha 2$ requires alternative methods. In the case of NH_2Y , two such methods have been considered. The first involves incorporation of $[\text{}^{14}\text{C}]\text{-NH}_2\text{Y}$ or $[\text{}^3\text{H}]\text{-NH}_2\text{Y}$ of known specific activity into $\alpha 2$ to determine the amount of NH_2Y by scintillation counting. A second method involves derivatization of NH_2Y with a known fluorophore and quantitation of the derivative by fluorescence spectroscopy (Hooker *et al.*, 2004). Both methods are currently being investigated.

The methods we have recently used to address the levels of contaminating wild-type $\alpha 2$ in our $\text{NH}_2\text{Y-}\alpha 2$ preparations consist of dCDP production assays in combination with the use of a stoichiometric mechanism-based inhibitor of RNRs, 2'-azido-2'-deoxyadenosine-5'-diphosphate (N_3ADP) (Thelander *et al.*, 1976). $\text{NH}_2\text{Y-}\alpha 2$ s were assayed for dCDP formation using the discontinuous radioactive assay with thioredoxin (TR), thioredoxin reductase (TRR), and NADPH as reductants. Our previous studies have shown that an activity $\{1/10^4\}$ that of wild-type RNR is the lower limit of detection by this assay (Seyedsayamdost and Stubbe, 2006; Yee *et al.*, 2003b). Observation of nucleotide reduction activity would suggest that either our $\text{NH}_2\text{Y-}\alpha 2$ preparations have a high level of Y incorporated at residues 730 (or 731) or that the $\text{NH}_2\text{Y-}\alpha 2$ s are inherently active in nucleotide reduction.

To distinguish between the two options, N_3ADP was used to quantitate the amount of wild-type $\alpha 2$. Previous studies have revealed that wild-type RNR is inactivated with 1 equivalent of N_3NDP , resulting in loss of 50% $\text{Y}_{122}\bullet$ (between 20 s and 2 min) and formation of 1 equivalent of a new substrate-derived nitrogen-centered radical ($\text{N}\bullet$, Fig. 3.5A) (Fritscher *et al.*, 2005; Salowe *et al.*, 1993; Sjöberg *et al.*, 1983; van der Donk *et al.*, 1995). The amount of $\text{N}\bullet$ is directly proportional to the amount of active RNR in solution (Ekberg *et al.*, 1998). Importantly, the EPR spectral features of $\text{N}\bullet$, $\text{Y}_{122}\bullet$, and $\text{NH}_2\text{Y}\bullet$ do not overlap in the low-field region, thereby allowing spectral deconvolution and quantitation of these three species (Fig. 3.5B).

6.8. Protocol for measurement of catalytic activities of $\text{NH}_2\text{Y-}\alpha 2$ s

The assay contained in a final volume of 230 μL : 0.2, 1 or 3 μM $\text{NH}_2\text{Y-}\alpha 2$, a 5-fold molar excess of $\beta 2$, 1 mM $[\text{5-}^3\text{H}]\text{-CDP}$ (1190 cpm/nmol, Amersham Bioscience), 3 mM ATP, 30 μM TR, 0.5 μM TRR, and 1 mM NADPH (Sigma-Aldrich) in assay buffer (50 mM Hepes, 15 mM MgSO_4 , 1 mM EDTA, pH 7.6) (Seyedsayamdost *et al.*, 2006b). The purification of TR (≈ 40 units/mg) (Chivers *et al.*, 1997), TRR (1400 units/mg) (Russel and Model, 1985), and $\beta 2$ (7200 nmol/min mg) (Salowe and Stubbe, 1986) have been described. For each

reaction, nucleotides, TR, TRR, and $\alpha 2$ were incubated at 25 °C for 1.5 to 2 min. The reaction was then initiated by addition of wild-type $\beta 2$ and NADPH. At defined time points, usually 0, 1, 2, 3, and 5 min, 40 μ L were withdrawn and quenched in 25 μ L of 2% (v/v) perchloric acid (Sigma-Aldrich). After the time course, the reactions were neutralized with 20 μ L of 0.5 M KOH (Fisher Bioscience). The samples were incubated at –20 °C overnight to ensure complete precipitation of potassium perchlorate. The samples were then thawed on ice and spun down for 3 min at 21,000g in a tabletop centrifuge. Each supernatant was transferred to a 1.5-mL screw-top microfuge tube, to which was added 14 units of calf-intestine alkaline phosphatase (Roche), 120 nmol of carrier deoxycytidine (Sigma-Aldrich), and final concentrations of 75 mM Tris and 0.15 mM EDTA (pH 8.5). The samples were incubated at 37 °C for 2 h and the amount [3 H]-dC in each sample was quantitated using the method of Steeper and Stuart (1970).

6.9. Protocol for use of the mechanism-based RNR inhibitor, N_3 ADP

The preparation of N_3 ADP and other N_3 NDPs, which may be used in this assay, has already been described (Hobbs and Eckstein, 1977; Robins *et al.*, 1992; Salowe *et al.*, 1987). The procedure for prereduction of $NH_2Y-\alpha 2$ s has also been reported (Seyedsayamdost *et al.*, 2007b). Prereduced $NH_2Y-\alpha 2$ (or wild-type $\alpha 2$) and dGTP (Sigma-Aldrich) were mixed with wild-type $\beta 2$ and N_3 ADP in assay buffer (50 mM Hepes, 15 mM $MgSO_4$, 1 mM EDTA, pH 7.6) to yield final concentrations of 20 μ M, 1 mM, 20 μ M, and 250 μ M, respectively. The mixture was transferred to an EPR tube (Wilmad) and the reaction quenched after 20 s by inserting the tube into liquid N_2 . EPR spectra were subsequently recorded at 77 K on a Bruker ESP-300 X-band spectrometer equipped with a quartz finger dewar filled with liquid N_2 . The EPR parameters were as follows: microwave frequency = 9.34 GHz, power = 30 μ W, modulation amplitude = 1.5 G, modulation frequency = 100 kHz, time constant = 5.12 ms, and scan time = 41.9 s. Analysis of the resulting spectra was carried out using an in-house written program in Excel and WinEPR (Bruker). These programs allow for accurate subtraction of the different spectral components in the reaction. In the experiments, deconvolution of the three signals observed ($Y_{122}\bullet$, $NH_2Y\bullet$, and $N\bullet$) was performed by first subtracting the $N\bullet$ spectrum, which is well established. Then, the well-characterized spectrum of unreacted $Y_{122}\bullet$ was subtracted, yielding the $NH_2Y-\alpha 2$ spectrum. The ratio of the three signals was determined by comparing the double integral intensity of each trace. The EPR spin quantitation was carried out using a Cu^{II} standard as has been described elsewhere (Palmer, 1967).

6.10. Results of activity and N_3 ADP assays

The activity assays for wild-type $\alpha 2$, $Y_{730}NH_2Y-\alpha 2$ and $Y_{731}NH_2Y-\alpha 2$ are summarized in Table 3.1. The results show that the $NH_2Y-\alpha 2$ variants retain significant nucleotide reduction activity. This activity may be inherent to $NH_2Y-\alpha 2$ s or may be attributed to wild-type endogenous $\alpha 2$ or wild-type $\alpha 2$ generated by mischarging of $mutRNA_{CUA}$ with Y rather than NH_2Y . To distinguish among the three options, N_3 ADP assays were carried out. As mentioned earlier, detailed characterization of wild-type $\alpha 2$ with N_3 NDPs by several groups has shown that after 20 to 120 s, 50 to 60% of the wild-type $\alpha 2$ population will generate the well-characterized $N\bullet$ spectrum. Therefore, if the residual activity observed is

due to wild-type $\alpha 2$, we would expect that 3.5% and 2% of $\alpha 2$ would contain the $N\bullet$ with $Y_{731}NH_2Y-\alpha 2$ and $Y_{730}NH_2Y-\alpha 2$, respectively.

The N_3ADP assays on $NH_2Y-\alpha 2$ s showed that for both $Y_{730}NH_2Y-\alpha 2$ and $Y_{731}NH_2Y-\alpha 2$, 15% of the initial $Y_{122}\bullet$ generates a $N\bullet$ (Table 3.1). This amount is significantly greater than the amount of $N\bullet$ expected, if its formation was due to wild-type $\alpha 2$. Therefore, the results indicate that the $NH_2Y-\alpha 2$ s are inherently active and capable of generating a $C_{439}\bullet$, which gives rise to the $N\bullet$. Further, they show that wild-type $\alpha 2$ levels in $NH_2Y-\alpha 2$ preparations are low, indicating specific and efficient incorporation of NH_2Y . As we have discussed before, the activity of $NH_2Y-\alpha 2$ s has major implications regarding the mechanism of radical propagation at residue Y_{730} (Seyedsayamdost *et al.*, 2007b). One problem with the N_3ADP assay is that $NH_2Y\bullet-\alpha 2$ is unstable on the time scale of the experiment resulting in loss of 20% of total spin. The other two radicals in this reaction, $Y_{122}\bullet$ and $N\bullet$, have been shown to be stable over 20 s. Thus, the $NH_2Y\bullet$ may partition between two pathways (Fig. 3.6). It may give rise to a $C_{439}\bullet$, which generates a $N\bullet$, or it may be reduced by components of the assay buffer. The latter pathway results in loss of spin.

Together, results from RNR activity and N_3ADP assays suggest that NH_2Y incorporation into $\alpha 2$ was efficient and specific; however, an accurate determination of the level of NH_2Y insertion will require one of the quantitative methods discussed in section 6.7.

7. Characterization of $NH_2Y-\alpha 2$ s

7.1. Overview

For NH_2Y to serve as a useful probe for enzymes that employ $Y\bullet$ s or modified $Y\bullet$ s in catalysis, its spectroscopic properties must be known. In this section, we describe the procedures we have used to characterize the EPR and UV-vis properties of $NH_2Y\bullet-\alpha 2$. We also present the protocols for determining the rate constant for $NH_2Y\bullet-\alpha 2$ formation using stopped flow (SF) UV-vis and rapid freeze-quench (RFQ) EPR spectroscopic methods.

7.2. Protocol for determining the EPR and UV-vis properties of $NH_2Y\bullet-\alpha 2$ s

To determine the EPR spectrum of $NH_2Y\bullet-\alpha 2$, prereduced $NH_2Y-\alpha 2$ and ATP were mixed with wild-type $\beta 2$ and CDP in assay buffer to give final concentrations of 20 to 24 μM , 3 mM, and 20 to 24 μM , 1 mM, respectively. The mixture was transferred to an EPR tube and quenched at defined time points by freezing in liquid N_2 . The EPR spectra were recorded at 77 K and analyzed as described in section 6.8.

To determine the absorption features of $NH_2Y_{730}\bullet$ and $NH_2Y_{731}\bullet$, SF UV-vis kinetics were carried out on an Applied Photophysics DX 17MV instrument equipped with the Pro-Data upgrade using PMT and diode array detection at 25 °C. Prereduced $Y_{730}NH_2Y-\alpha 2$ (or $Y_{731}NH_2Y-\alpha 2$) and ATP in one syringe were rapidly mixed in a 1:1 ratio with wild-type $\beta 2$ and CDP from a second syringe to yield final concentrations of 8 to 10 μM , 3 mM, and 8 to 10 μM , 1 mM, respectively, in assay buffer. Initially, data was collected with a PDA.1 diode array detector by recording a spectrum between 300 and 700 nm every 100 ms after mixing. After identifying the spectral region at which $NH_2Y\bullet$ absorbs, the reaction was repeated using PMT detection to obtain a more accurate absorption profile of $NH_2Y\bullet-\alpha 2$. Two to four

traces were recorded on a 1.5 to 2 s time scale and averaged between 305 and 365 nm in 5-nm intervals. The absorption change was corrected for the absorption of $Y_{122}^{\bullet}$ in this region, given its well-characterized ϵ at these λ s, (Bollinger *et al.*, 1995; Gräslund *et al.*, 1985; Nyholm *et al.*, 1993) and then plotted against λ . Calculation of the ϵ for $Y_{730}NH_2Y^{\bullet}-\alpha 2$ ($10,500\text{ M}^{-1}\text{cm}^{-1}$) and $Y_{731}NH_2Y^{\bullet}-\alpha 2$ ($11,000\text{ M}^{-1}\text{cm}^{-1}$) was performed using the ϵ of $Y_{122}^{\bullet}$ ($\epsilon_{410\text{ nm}} = 3,700\text{ M}^{-1}\text{cm}^{-1}$) (Bollinger *et al.*, 1995), assuming that consumption of each mole of $Y_{122}^{\bullet}$ leads to formation of 1 mole of $NH_2Y^{\bullet}$ in $\alpha 2$. Curve fitting was performed with OriginPro or KaleidaGraph Software.

7.3. Results

To monitor changes in the concentration of $NH_2Y^{\bullet}$, its UV-vis and EPR spectral features must first be determined. These properties have facilitated our studies on $NH_2Y-\alpha 2$ s and will aid other research groups that intend to use NH_2Y as a probe. The reaction of $NH_2Y-\alpha 2/\beta 2$ with CDP/ATP, monitored by EPR spectroscopy, is shown in Fig. 3.7. The observed spectrum (Fig. 3.7A, black trace) is a mixture of two species: $Y_{122}^{\bullet}$ and $NH_2Y^{\bullet}$. The distinct low-field features of $Y_{122}^{\bullet}$ were used to subtract its contribution from the observed spectrum to reveal the red trace in Fig. 3.7A, the spectrum of $NH_2Y_{731}^{\bullet}$. It consists of a fairly isotropic signal with a g value of 2.0043 as expected for an organic radical. In the absence of substrate and effector, only the $Y_{122}^{\bullet}$ is observed (Fig. 3.7B). This is an important control indicating pathway-dependent formation of $NH_2Y^{\bullet}$ only when substrate and effector are bound to the $NH_2Y-\alpha 2/\beta 2$ complex. Interestingly, the spectra of $NH_2Y_{730}^{\bullet}$ and $NH_2Y_{731}^{\bullet}$ are similar but distinct, perhaps reflecting differences in the conformation(s) and/or environment around residue 730 vs. 731 (Fig. 3.7C).

Next, we wished to characterize the UV-vis features of $NH_2Y^{\bullet}$. We hypothesized that its absorption profile would be similar to that of DOPA $^{\bullet}$ (Craw *et al.*, 1984), given the structural similarity between the two species. SF diode array spectroscopy clearly showed changes in the region between 310 and 365 nm (data not shown). The extinction coefficients associated with $Y_{122}^{\bullet}$ between 310 and 365 nm are known and small ($\epsilon \approx 500$ to $1900\text{ M}^{-1}\text{cm}^{-1}$) (Bollinger *et al.*, 1995; Gräslund *et al.*, 1985; Nyholm *et al.*, 1993) and can be used in spectral deconvolution. To determine the features of $NH_2Y^{\bullet}$ more accurately, SF UV-vis was carried out using PMT detection. The absorbance change at 1.5 s at each λ , corrected for the absorption by the $Y_{122}^{\bullet}$, was plotted against the λ . The results are shown in Fig. 3.8A and indicate that $NH_2Y_{730}^{\bullet}$ and $NH_2Y_{731}^{\bullet}$ have similar absorption profiles, which resemble the profile of DOPA $^{\bullet}$ (Craw *et al.*, 1984). The UV-vis spectrum of $NH_2Y_{730}^{\bullet}$ consists of a broad feature with a λ_{max} at 325 nm ($\epsilon \approx 10,500\text{ M}^{-1}\text{cm}^{-1}$). The $NH_2Y_{731}^{\bullet}$ spectrum exhibits a λ_{max} at 320 nm ($\epsilon \approx 11,000\text{ M}^{-1}\text{cm}^{-1}$) and a more defined shoulder at 350 nm.

7.4. Protocol for determining the kinetics of $NH_2Y^{\bullet}-\alpha 2$ formation by SF UV-vis spectroscopy

To determine the kinetics of $Y_{122}^{\bullet}$ consumption and $NH_2Y^{\bullet}-\alpha 2$ formation, a reaction identical to the one described previously was carried out. At least six individual traces were averaged at 410 nm (λ_{max} of $Y_{122}^{\bullet}$) and 325 nm (λ_{max} of $NH_2Y_{730}^{\bullet}$) or 320 nm (λ_{max} of $NH_2Y_{731}^{\bullet}$). Curve fitting was performed with OriginPro or KaleidaGraph Software. To

obtain kinetic constants, iterative rounds of fitting and calculation of a residual plot and the R^2 correlation value were carried out until both were optimized.

7.5. Protocol for determining the kinetics of $\text{NH}_2\text{Y}\cdot\text{-}\alpha 2$ formation by RFQ EPR spectroscopy

Rapid freeze-quench EPR samples were prepared using an Update Instruments 1019 Syringe Ram Unit, a Model 715 Syringe Ram Controller, and a quenching bath (which consists of a large outer stainless-steel pot that contains a smaller $\approx 7\text{-L}$ liquid isopentane bath). The temperature of the liquid isopentane bath was controlled with a Fluke 52 Dual Input Thermometer, equipped with an Anritsu Cu Thermocouple probe for the isopentane bath and the funnel. Stainless-steel packers were purchased from McMaster-Carr and were cut to a length of 40 cm and deburred at the MIT machine shop. The dead-time of the setup was determined to be 16 ± 2 ms with two independent measurements of the myoglobin/ NaN_3 test reaction according to previously published procedures (Ballou and Palmer, 1974). A packing factor of 0.60 ± 0.05 was reproducibly obtained as tested with wild-type $\beta 2$ samples. Routinely, a ram push velocity of 1.25 or 1.6 cm/s was used, and the displacement was adjusted to expel 300 μL of sample after the reaction.

Operation of the apparatus was similar to the procedures previously described (Ballou and Palmer, 1974; Bollinger *et al.*, 1995). Briefly, the temperature of the isopentane bath was lowered to -135 to -140 $^\circ\text{C}$ with liquid N_2 in the outer bath. One syringe contained 38 μM prereduced $\text{Y}_{731}\text{NH}_2\text{Y}\text{-}\alpha 2$ and 6 mM ATP in assay buffer and was mixed with 38 μM wild-type $\beta 2$ and 2 mM CDP in assay buffer from a second syringe. When the temperature of the EPR tube-funnel assembly had equilibrated to the bath temperature, the contents of each syringe were mixed rapidly and aged for a predetermined period by traversing the contents through a reaction loop. The sample was then sprayed into the EPR tube-funnel assembly, which was held at a distance of 1 cm from the spray nozzle. After the sample was sprayed into the funnel, the assembly was immediately returned to the bath and the crystals allowed to settle for 15 to 30 s. The sample was then packed into the EPR tube using the stainless-steel packers. After packing, excess isopentane was removed by flicking the EPR tube or by using a Pasteur pipette. The EPR tube was stored in liquid N_2 until the EPR spectrum was recorded. Acquisition of EPR spectra was carried out as described in section 6.8. Each time point was collected in duplicates. The quenching times, including the instrument dead time, are indicated in the figure legend.

7.6. Results of SF UV-vis and RFQ EPR spectroscopic studies

The SF UV-vis experiments were carried out to monitor the kinetics of $\text{Y}_{122}\cdot$ disappearance and $\text{NH}_2\text{Y}_{731}\cdot$ formation. The results are shown in Fig. 3.8B. At 410 nm, biexponential kinetics with rate constants of 17.3 ± 0.2 and 2.3 ± 0.1 s^{-1} were observed for loss of $\text{Y}_{122}\cdot$, similar to rate constants obtained for formation of $\text{NH}_2\text{Y}_{731}\cdot$ at 320 nm (21.0 ± 0.1 s^{-1} and 3.0 ± 0.1 s^{-1}). Analogous experiments carried out with $\text{Y}_{730}\text{NH}_2\text{Y}\text{-}\alpha 2$ showed that disappearance of $\text{Y}_{122}\cdot$ occurred biexponentially (12.0 ± 0.1 s^{-1} and 2.4 ± 0.1 s^{-1}), concomitant with formation of $\text{NH}_2\text{Y}_{730}\cdot$ (13.6 ± 0.1 s^{-1} and 2.5 ± 0.1 s^{-1} , data not shown). A control experiment was carried out in the absence of substrate and effector with $\text{Y}_{731}\text{NH}_2\text{Y}\text{-}\alpha 2$ (or $\text{Y}_{730}\text{NH}_2\text{Y}\text{-}\alpha 2$) and $\beta 2$. As indicated by EPR experiments, no loss of $\text{Y}_{122}\cdot$ or formation of $\text{NH}_2\text{Y}\cdot$ occurred under identical conditions (data not shown).

The slow and fast rate constants for $\text{NH}_2\text{Y}\cdot\text{-}\alpha 2$ formation both occur in a fashion that is kinetically competent to be involved in dNDP formation. Thus, studies with the mutants provide the first direct evidence for their involvement in radical propagation. The slow rate constants, also observed in the DOPA- $\beta 2$ experiments, are similar to the steady-state rate constant for RNR turnover. See Seyedsayamdost *et al.* (2007b) for further discussion of the observed rate constants and amplitudes.

The RFQ-EPR data monitoring formation of $\text{NH}_2\text{Y}_{731}\cdot$ is shown in Fig. 3.9. A double exponential fit to the data is not warranted because fewer data points are accessible by this method, relative to SF UV-vis spectroscopy. We have therefore used the SF UV-vis data to fix the rate constant and amplitude of the slow phase in our double exponential fitting procedure of the RFQ-EPR data. The fit then yields the rate constant and amplitude of the fast phase, which are $19.2 \pm 2.4 \text{ s}^{-1}$ and $24 \pm 2\%$, respectively, in excellent agreement with the dominant kinetic phase in the SF UV-vis experiments. The total amplitude observed in this reaction, $35 \pm 4\%$ $\text{NH}_2\text{Y}_{731}\cdot$ relative to $[\text{Y}_{122}\cdot]$ at $t = 0$, also agrees with the total amplitude for $\text{NH}_2\text{Y}_{731}\cdot$ formation from the SF UV-vis studies ($35 \pm 1\%$ $\text{NH}_2\text{Y}_{731}\cdot\%$).

8. Summary

Studies using the evolved suppressor tRNA/RS pair to incorporate NH_2Y site-specifically into RNR show the power of this method to study mechanistic issues that are not accessible by any other methods currently available. Site-directed mutagenesis, a universally used method, took decades to evolve into the essential technology it is today. Likewise, incorporation of unnatural amino acids in a similarly robust fashion will require the efforts of many investigators and the sharing of methodological improvements. The radical propagation reaction catalyzed by RNR over $35 \text{ \AA}$ between two protein subunits is unprecedented. Only with the use of NH_2Y and other evolved suppressor tRNA/RS pairs (e.g. for fluorotyrosines (Seyedsayamdost *et al.*, 2006a) and 3-nitrotyrosine (Neumann *et al.*, 2008; Yee *et al.*, 2003b)) will the mechanistic details of this remarkable reaction become unveiled.

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Figure 3.1.

The radical transfer pathway in *E. coli* RNR. Residues Y₁₂₂, W₄₈, E₂₃₇, and Y₃₅₆ reside in $\beta 2$, while Y₇₃₁, Y₇₃₀, C₄₃₉, and E₄₄₁ reside in $\alpha 2$. Residues in gray have been shown to be redox-active using DOPA- $\beta 2$ and NH₂Y- $\alpha 2$ s (see text). Note that the position of Y₃₅₆ is unknown from structural studies.

General scheme for selection of $\text{NH}_2\text{Y-RS}$. In the positive selection, suppression of a permissive TAG codon in chloramphenicol acetyl transferase (CAT, the Cm resistance gene) allows synthetases that charge $\text{mutRNA}_{\text{CUA}}$ with any amino acid to survive. The positive selection may also be performed by monitoring green fluorescence stemming from GFPuv, which contains a T7 promoter and is expressed when the TAG codons in the T7 RNA polymerase gene are suppressed. The synthetases are then carried through a negative selection cycle in which those that suppress the TAG codons in the barnase gene in the absence of NH_2Y are eliminated. Therefore, only synthetases that are functional with the host's translation machinery and do not charge $\text{mutRNA}_{\text{CUA}}$ with a natural amino acid will survive. See text for a description of the features on each plasmid.

Figure 3.3.

SDS-PAGE and MALDI-TOF MS analysis of K_7NH_2Y -Z-domain. (A) SDS gel of purified Z-domain after expression in the absence (lane 2) or presence (lane 3) of NH_2Y . The arrow designates the band corresponding to K_7NH_2Y -Z-domain. Protein ladder and MWs are shown in lane 1. (B) MALDI-TOF MS of purified K_7NH_2Y -Z-domain obtained under positive ionization mode. For the four main peaks in the spectrum, m/z $[M+H]^+$ are indicated. These correspond to N-terminally cleaved Met form of K_7NH_2Y -Z-domain, $[M+H]^+_{exp} = 7814$; its acetylated form, $[M+H]^+_{exp} = 7856$; full-length K_7NH_2Y -His-Z-domain, $[M+H]^+_{exp} = 7945$; and its acetylated form, $[M+H]^+_{exp} = 7987$.

Figure 3.4. Incorporation of NH₂Y into $\alpha 2$. Expression of Y₇₃₁NH₂Y- $\alpha 2$ (A) and Y₇₃₀NH₂Y- $\alpha 2$ (B) are shown as a function of IPTG, NH₂Y/DTT and temperature. Cells were grown in the presence or absence of IPTG and NH₂Y/DTT 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. For both constructs, no expression is observed in the absence of IPTG and expression of truncated $\alpha 2$ is observed in the absence of NH₂Y. Full-length NH₂Y- $\alpha 2$ is seen only in the presence of IPTG and NH₂Y/DTT.

Figure 3.5.

(A) Structure of the active site N-centered radical formed after reaction of RNR with N_3ADP . This radical is formed at the expense of $\text{Y}_{122}\bullet$. It is stable on the minute time scale and is abbreviated as $\text{N}\bullet$ in the text. (B) EPR spectra of $\text{N}\bullet$ (green), $\text{Y}_{122}\bullet$ (blue), and $\text{NH}_2\text{Y}_{731}\bullet$ (red). The distinct features in the low-field region between ≈ 2.02 and ≈ 2.03 were used to perform subtractions and quantitations of the three species in N_3ADP assays.

Figure 3.6.

Working model for loss of spin during reaction of NH_2Y - $\alpha 2s$ with $N_3ADP/dGTP$. After radical initiation and $NH_2Y^{\bullet}$ formation (k_A), partitioning occurs between $C_{439}^{\bullet}$ formation and subsequent $N^{\bullet}$ formation (k_C) or quenching by yet unknown mechanisms (k_B).

Figure 3.7.

Reaction of NH₂Y-α₂/ATP with wild-type β₂/CDP monitored by EPR spectroscopy. (A) The reaction components were mixed at 25 °C to yield final concentrations of 20 μM Y₇₃₁NH₂Y-α₂/β₂ complex, 1 mM CDP, and 3 mM ATP in assay buffer. After 20 s, the reaction was quenched by hand-freezing in liquid N₂ and the EPR spectrum recorded at 77 K. Unreacted Y₁₂₂• (blue trace, 58% of total spin) was subtracted from the observed spectrum (black trace) to reveal the spectrum of NH₂Y₇₃₁• (red trace, 42 % of total spin). (B) Reaction of Y₇₃₁NH₂Y-α₂/β₂ in the absence of CDP/ATP. Only the Y₁₂₂• spectrum is observed in this case. (C) Reaction of Y₇₃₀NH₂Y-α₂ with β₂ and CDP/ATP, similar to that described in (A). The subtracted spectrum of NH₂Y₇₃₀• is shown.

Figure 3.8.

Characterization of $\text{NH}_2\text{Y-}\alpha 2\text{s}$ by SF UV-vis spectroscopy. (A) Point-by-point reconstruction of the UV-vis spectrum of $\text{NH}_2\text{Y}_{730}\bullet$ (blue dots) and $\text{NH}_2\text{Y}_{731}\bullet$ (red dots). Prerduced $\text{Y}_{730}\text{NH}_2\text{Y-}\alpha 2$ and ATP in one syringe were mixed with wild-type $\beta 2$ and CDP from another syringe, yielding final concentrations of $10\ \mu\text{M}$, $3\ \text{mM}$, $10\ \mu\text{M}$, and $1\ \text{mM}$, respectively. With $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$, the reaction was carried out at final concentrations of $9\ \mu\text{M}$ $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2/\beta 2$, $1\ \text{mM}$ CDP, and $3\ \text{mM}$ ATP. The absorption change was monitored in 5-nm intervals; at each λ , 2 to 4 time courses were averaged and corrected for the absorption of $\text{Y}_{122}\bullet$ using previously determined ϵ in this spectral range. The corrected OD was converted to ϵ , which was then plotted against λ . (B) Prerduced $\text{Y}_{731}\text{NH}_2\text{Y-}\alpha 2$ and CDP in one syringe were mixed in a 1:1 ratio with $\beta 2$ and ATP from another syringe to yield the same concentrations as in (A). The concentration of $\text{Y}_{122}\bullet$ (blue trace) and $\text{NH}_2\text{Y}_{731}\bullet$ (red trace) were monitored at 410 nm and 320 nm, respectively. A total of 6 traces were averaged at each λ . See text for kinetic parameters.

Figure 3.9.

Analysis of $\text{NH}_2\text{Y}_{731}^\bullet$ formation by rapid-freeze quench EPR spectroscopy. (A) $\text{Y}_{731}\text{NH}_2\text{Y}-\alpha 2$ and ATP were rapidly mixed with wild-type $\beta 2$ and CDP to yield final concentrations of $19 \mu\text{M}$ $\beta 2/\text{Y}_{731}\text{NH}_2\text{Y}-\alpha 2$, 3 mM ATP and 1 mM CDP. The reaction was quenched at various time points by spraying its contents into a liquid isopentane bath maintained at $\approx -140^\circ\text{C}$. The EPR spectra were subsequently recorded at 77 K . Shown are the spectra at 0 ms (black), 72 ms (red), 312 ms (blue), and 1.16 s (green). (B) Kinetic trace obtained after subtracting the $\text{Y}_{122}^\bullet$ component from each trace and plotting the percentage of $\text{NH}_2\text{Y}_{731}^\bullet$ (relative to total spin) versus time. In this double exponential fit, the rate constant and amplitude for the slow phase have been held constant using the values from the SF UV-vis studies. Therefore, the fit yields the parameters for the fast kinetic phase. See text for kinetic parameters.

Table 3.1Activities of wild-type $\alpha 2$ and NH₂Y- $\alpha 2$ s using radioactive product formation and N₃ADP assays

$\alpha 2$ variant	Radioactive RNR assay (% wt) ^a	N ₃ ADP assay (% N• at 20 s vs. initial Y ₁₂₂ •) ^b
wt $\alpha 2$	100 ^a	52
Y ₇₃₀ NH ₂ Y- $\alpha 2$	4 ± 0.5	15 ± 2 ^b
Y ₇₃₁ NH ₂ Y- $\alpha 2$	7 ± 0.5	15 ± 2 ^b

^aThe activities are reported as percentage of wild-type $\alpha 2$ activity, which was 2500 nmol/min mg. The error is the standard deviation from 2 independent measurements.

^bNote that the amount of N• is reported relative to [Y₁₂₂•] at t = 0; the error is associated with EPR spin quantitation.