Metal-free class Ie ribonucleotide reductase from pathogens initiates catalysis with a tyrosine-derived dihydroxyphenylalanine radical

Elizabeth J. Blaesef,1, Gavin M. Palowitcb,1, Kai Huc,d,1, Amelia J. Kimb,1, Hannah R. Rosef, Rahul Alapati1, Marshall G. Loughee, Hee Jong Kim4, Alexander T. Taguchig, Kong Ooi Tang, Tatiana N. Laremorec, Robert G. Griffin9, Carsten Krebsab,b,2, Megan L. Matthewsfe,f,2, Alexey Silakovoa,b,2, J. Martin Bollinger Jr.ab,b,2, Benjamin D. Allenb,c,2, and Amie K. Boala,b,2

aDepartment of Chemistry, The Pennsylvania State University, University Park, PA 16802; bDepartment of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802; cHuck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802; dMolecular, Cellular, and Integrative Biosciences Graduate Program, The Pennsylvania State University, University Park, PA 16802; eDepartment of Chemistry, University of Pennsylvania, Philadelphia, PA 19104; fBiochemistry and Molecular Biophysics Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; and gDepartment of Chemistry, Francis Bitter Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139

Edited by JoAnne Stubbe, Massachusetts Institute of Technology, Cambridge, MA, and approved August 20, 2018 (received for review July 13, 2018)

All cells obtain 2'-deoxyribonucleotides for DNA synthesis through the activity of a ribonucleotide reductase (RNR). The class I RNRs found in humans and pathogenic bacteria differ in (i) use of Fe(II), Mn(II), or both for activation of the dinuclear-metallocofactor subunit; (ii) (ii) reaction of the reduced dinuclear metal center with dioxygen or superoxide for this activation; (iii) requirement (or lack thereof) for a flavoprotein activase, NrdI, to provide the superoxide from O2; and (iv) use of either a stable tyrosyl radical or a high-valent metallocluster to initiate each turnover by oxidizing a cysteine residue in the α subunit to a radical (Cys•). The use of manganese by bacterial class I, subclass b-d RNRs, which contrasts with the exclusive use of iron by the eukaryotic la enzymes, appears to be a countermeasure of certain pathogens against iron deprivation imposed by their hosts. Here, we report a metal-free type of class I RNR (subclass e) from two human pathogens. The Cys• in its α subunit is generated by a stable, tyrosine-derived dihydroxyphenylalanine radical (DOPA•) in β. The three-electron oxidation producing DOPA• occurs in Escherichia coli only if the β is coexpressed with the NrdI activase encoded adjacently in the pathogen genome. The independence of this new RNR from transition metals, or the requirement for a single metal ion only transiently for activation, may afford the pathogens an even more potent countermeasure against transition metal-directed innate immunity.

DNA biosynthesis | semiquinone | DOPA

Ribonucleotide reductases (RNRs) catalyze the only known biochemical reaction—replacement of the 2'-hydroxyl group of a ribonucleoside 5’-diphosphate or -triphosphate by hydrogen—that provides the substrates for DNA replication and repair (1). RNRs use a largely conserved pathway for C2’ reduction. A transient thyl radical produced by one-electron oxidation of a cysteine residue (Cys•) removes a hydrogen atom (H•) from C3’ of the substrate (2). The 3 radical activates C2’ for dehydration, and ensuing electron, proton, and/or hydrogen-atom transfers (from sulfur-containing amino acids and/or formate) then generate a 2'-deoxy 3'-radical, which retakes the H• from the cysteine to complete the reduction and regenerate the Cys• (3). Importantly, the Cys• is not present in any resting RNR but must be generated at the outset and quenched at the end of each turnover. The imperative for Cys• production engendered at least three distinct classes (I–III) of RNRs and four known subclasses (a–d) within class I (1, 4, 5). Different transition metal-dependent strategies for Cys• generation define the (sub)classes. Class II (6) and class III (7) RNRs use cobalamin and iron-sulfur cofactors, respectively, to generate the Cys•. The class III enzymes undergo preactivation by a separate protein, a radical-SAM (S-adenosyl-l-methionine) activase (7). The activase uses its iron-sulfur cluster to reductively cleave SAM, and the resultant 5'-dA abstracts H• from a glycine residue in the RNR. The stable glycylic radical generates the Cys• to initiate each turnover.

Class I RNRs, such as the enzymes found in all eukarya and many pathogenic bacteria, use a separate ferritin-like protein subunit, denoted β, as radical initiator. RNR β subunits contain four core helices that provide six metal ligands, two histidines and four carboxylates (Fig. 1 A and B), to a dinuclear, divalent metal cluster (M2III(III)) (4). M2III(III) •-reacts with a form of dioxygen to install a stable oxidant that generates the Cys• in the catalytic subunit, α, by a long-range intersubunit radical-translocation (RT) process among the four core helices.

Significance

Conversion of ribonucleotides to the 2'-deoxyribonucleotides required for DNA biosynthesis is catalyzed by ribonucleotide reductases (RNRs) via a free-radical mechanism. Known types of RNRs all depend on redox-active transition metals—manganese, iron, or cobalt—for radical initiation. Pathogenic bacteria are challenged by transition metal sequestration and infliction of oxidative stress by their hosts, and the deployment of multiple RNRs with different metal requirements and radical-initiating oxidants is a known bacterial countermeasure. A class I RNR from two bacterial pathogens completely lacks transition metals in its active state and uses a tyrosine-derived dihydroxyphenylalanine radical as its initiator, embodying a novel tactic to combat transition metal- and oxidant-mediated innate immunity and reinforcing bacterial RNRs as potential antibiotic targets.
mediated by a chain of conserved aromatic residues that spans the subunit interface and connects the active sites (8). RT is bidirectional, occurring in the first and last steps of each turnover to generate and quench the 3'-H-cleaving Cys.

The identities of the transition metal complexes (manganese or iron), activating oxidants (dioxygen or superoxide), and Cys generators (high-valent dimetal center or tyrosyl radical) are the basis for the division of class I enzymes into subclasses a–d (4, 5). The Ib and Id enzymes use their dimetal clusters (Fe and Mn, respectively) to oxidize a nearby tyrosine residue to a stable radical (Tyr•), which serves as the catalytic initiator. The Ic and Id enzymes use a stable, oxidized form of the dimetal cluster itself (Mn/Fe and Mn, respectively) directly for Cys generation. The presence of Fe in the Ia and Ib proteins allows them to undergo activation by O2; this reaction converts the Fe••/Fe•• complex to their Fe••••/Tyr• and Fe••/Fe•• active states. The Mn•••• complexes of the Ib and Id βs are unreactive toward O2 and must instead be oxidized by superoxide. For the Ib β, the superoxide is provided by a flavoprotein activase, NrdL, that binds to β and forms a hydrophilic channel connecting their active sites. The NrdL flavin reduces O2, and the channel guides the enzyme to the Mn••••• cluster in β. The resulting reaction generates the Mn•••••/Tyr• active state. The β subunit of the Id RNR from Flavobacterium johnsoniae (Fj) can scavenge superoxide directly from solution to oxidize the Mn••••• complex to the active Mn••••• state (5). Accordingly, the genomes of Fj and other organisms with Id RNRs do not encode NrdL homologs.

The RNR(β) of an organism equips it for its environmental niche (9, 10). For example, both the glycol radical of a class III RNR and the iron-sulfur cluster of its activase are unstable in the presence of O2, limiting the enzymes to anaerobes, whereas class I RNRs require O2 for activation and are functional only in aerobes, including all eukaryotes. Escherichia coli (Ec) has both types, expressing its class III enzyme during anaerobic growth and its class Ia enzyme during aerobic growth. Analogously, whereas eukaryotes possess exclusively iron-dependent class I enzymes, the use of manganese-dependent class I (b–d) RNRs appears to equip bacteria to cope with stresses encountered in their more dynamic niches. For example, the class Ib Ec enzyme sustains replication only under conditions of iron limitation or oxidative stress (11, 12). Thus, Ec mutants with a nonfunctional class Ia RNR subunit are not viable in the presence of O2 under normal growth conditions, and an Ec strain with a mutation that renders its class Ia subunit inactive at elevated temperatures (42 °C) has been used as a functional selection for heterologous RNRs (13, 14). The facts that (i) the immune systems of warm-blooded hosts inflict both iron limitation and oxidative stress on an invading bacterium (15) and (ii) class Ib enzymes are highly represented in pathogens (16) have led to the hypothesis that the use of manganese is a specific countermeasure against iron-directed innate immunity. Consistent with this notion, Streptococcus pneumoniae, an opportunistic pathogen that is normally present in the human mouth but can be unleashed by dental procedures to cause subacute bacterial endocarditis, requires its class Ib RNR, but not its class III enzyme, for virulence in an animal model of the disease (17, 18). It has been suggested that the divergent metal requirements of the human and bacterial RNRs could be leveraged for antibiotic development (18).

In view of the likely relevance of the diverse radical-initiation chemistry of RNRs to bacterial pathogenesis, we continue to mine sequence databases for orthologs that deploy hitherto unknown strategies. We show here that the β subunits of a pair of class I RNRs from two human pathogens, Streptococcus pyogenes (Sp) (19) and Aerococcus urinae (Au) (20), lack any transition metal in their active states and use as their initiator a previously unknown stable dihydroxyphenyalanine radical (DOPA••) derived posttranslationally from tyrosine in an NrdL- and O2-dependent reaction. These proteins are representative of a larger sequence group of class I RNR βs (class Ie) that are expected to function in the same way and are prevalent in parasitic and commensal organisms (SI Appendix, Fig. S1). Although the Ie βs might still require a single, as-yet unidentified transition metal ion transiently for activation, in either case they would exemplify the most sparing metal use of any known RNR and thus could afford the most effective RNR-based countermeasure yet discovered against transition metal restriction by the host.

Results and Discussion

A Group of Active Class I RNRs Lacking Three Glu Metal Ligands in β. To identify RNRs with unusual cofactors or activation mechanisms, we compiled β sequences (14,807 unique UniProt queries, July 2018) and generated a multiple sequence alignment to mine computationally for examples that fail to conserve residues with established functions (radical Tyr). Megalopolitan analysis of a group of 430 sequences all lack three otherwise-conserved metal-coordinating glutamates (Fig. 1 A and B, light blue), which are replaced by two hydrophobic residues and a cationic Lys. These β sequences are adjacent to the Ib subclass in molecular phylogeny (Fig. 1C), and, accordingly, almost all of them are encoded close to genes for apparent NrdL activases. Importantly, the associated operon (encoding β-NrdL-α) from Sp, the causative agent of strep throat and scarlet fever, can complement the aforementioned Ia RNR-a temperature-sensitive mutant at the restrictive temperature, as shown by Sjöberg et al. (14), thereby ruling out the possibility that they are nonfunctional, vestigial sequences. We verified that synthetic operons with all three components from either Sp or Au give robust complementation in this assay (SI Appendix, Table S1). We also found that the NrdL component from Au can functionally substitute for Sp NrdL in supporting the in vivo function of the Sp RNR.

To understand how these β proteins can be active despite lacking three metal ligands present in all other orthologs, we expressed (in Ec) N-terminally affinity-tagged Sp and Au β subunits with and without their NrdL activases and purified them chromatographically (SI Appendix, Fig. S2). We also expressed and purified affinity-tagged versions of the cognate α subunits for RNR activity measurements. The β subunits purified following coexpression with an NrdL, including the Sp β coexpressed with the Au activase, were capable of supporting reduction of CDP to dCDP in the presence of the cognate α subunit and DTT (SI Appendix, Figs. S3 and S4).
Maximal turnover rates of 0.35 s⁻¹·p⁻¹ were observed (SI Appendix, Fig. S3) for the Sp β. In contrast, preparations of β proteins obtained following expression in E. coli in the absence of a NrdI undetectable activity (<2 × 10⁻⁵ s⁻¹·p⁻¹). The Sp-β/α-NrdI coexpression system more readily yielded β with minimal contaminating NrdI, with its intensely absorbing flavin (Fig. 2A and SI Appendix, Fig. S3); thus, the heterologous system was used to generate the active protein for the spectroscopic analysis presented below.

The Active Class Ie βs Lack Metal and Contain a Dihydroxyphenylalanine Radical (DOPA⁺). Preparations of activated (NrdI-coexpressed) Sp and Au βs exhibited a broad absorption feature at 690 nm and a complex of four sharper peaks at approximately 383, 364, 349, and 337 nm (Fig. 2A and SI Appendix, Fig. S4), which were absent from the preparations of unactivated (expressed alone) proteins. The sharpest peak of the complex (383 nm) resembles the hallmark feature of a Tyr•, but its wavelength is less than that for any Tyr• in a class Ia/b RNR (405 nm). Use of protein containing [2H₂]-Tyr caused a narrowing characteristic of the replacement of a strong doublet narrowig feature of a Tyr•-derived radical by dithionite. (SI Appendix, Table S3) of activated and unactivated forms of Au β, which formed high-quality crystals more readily than the Sp β protein. Structures solved at 1.6-A resolution showed no density attributable to a metal ion in either form (Fig. 3A and SI Appendix, Fig. S7) and confirmed that the substitution of the three Glu ligands is not compensated for by a change in protein fold that introduces other ligands to preserve metal site 2. Moreover, the structures revealed that the ammonium group of the Lys that replaces a Glu ligand comes to occupy the usual location of the site 1 metal ion (SI Appendix, Fig. S8) (27–29), thus rationalizing the complete absence of metal. Exclusively in the structure of the activated form, clear Fe₃⁺/Fe²⁺ difference density could be observed near one of the carbons (C₅) ortho to the hydroxyl group of Tyr123 (SI Appendix, Fig. S7B), revealing hydroxylation of Tyr123 to dihydroxyphenylalanine (DOPA) in the active state and garnering insight into the structure of the Tyr-derived radical as the initiator for the new metal-independent after activation in vivo.

We verified the absence of any transition metal in the active state and garnered insight into the structure of the Tyr-derived radical by X-ray crystallographic analysis (SI Appendix, Table S3) of activated and unactivated forms of Au β, which formed high-quality crystals more readily than the Sp β protein. Structures solved at 1.6-A resolution showed no density attributable to a metal ion in either form (Fig. 3A and SI Appendix, Fig. S7) and confirmed that the substitution of the three Glu ligands is not compensated for by a change in protein fold that introduces other ligands to preserve metal site 2. Moreover, the structures revealed that the ammonium group of the Lys that replaces a Glu ligand comes to occupy the usual location of the site 1 metal ion (SI Appendix, Fig. S8) (27–29), thus rationalizing the complete absence of metal. Exclusively in the structure of the activated form, clear Fe₃⁺/Fe²⁺ difference density could be observed near one of the carbons (C₅) ortho to the hydroxyl group of Tyr123 (SI Appendix, Fig. S7B), revealing hydroxylation of Tyr123 to dihydroxyphenylalanine (DOPA) in the active state and garnering insight into the structure of the Tyr-derived radical as the initiator for the new metal-independent after activation in vivo.

We next used mass spectrometry to characterize the Tyr123 modification. Initial whole-protein analysis of Au and Sp β suggested gains in mass of 15.7 ± 0.7 Da and 15.5 ± 0.6 Da, respectively, on activation (SI Appendix, Table S4), consistent with the +16-Da change expected for hydroxylation. Proteolysis of activated and unactivated forms of both Sp and Au β (sequentially treated with trypsin and endopeptidase GluC), chromatographic separation of peptides, and sequencing by high-resolution mass-spectrometric fragmentation (LC-MS/MS) provided data in support of the site of modification and the chemical identity deduced by crystallography (Fig. 3B). An unbiased search (30) of all MS2 spectra for four or more of the y-ions expected to result from the predicted 13-mer peptide containing Tyr123 allowed its identification in both activated and unactivated preparations. The masses of the dominant ions from the activated Sp and Au β preparations were within 1.0 and 1.7 ppm of those predicted for the Cys-carbamidomethylated, Tyr123-hydroxylated 13-mer in its quinone form ([M+H]⁺ = 1,465,616 Da for Sp and 1,479,633 Da for Au) (Fig. 3B and SI Appendix, Fig. S10 and Table S5). Observation of the quinone, rather than DOPA itself, is expected, given that the analysis was performed in air. For the Sp protein, an additional modification, consistent with iodination of the DOPA123 quinone during Cys-iodination by excess iodacetamide, was also observed (SI Appendix, Fig. S10). In neither system could a Tyr123-modified peptide be detected in the unactivated β sample expressed without NrdI.

Without tuning by its active-site environment (e.g., by control of protonation state), the DOPA radical (DOPA⁺) implicated by the crystallographic and LC-MS/MS data would not be expected to have a reduction potential sufficient for its generation of Cys• (31, 32). Thus, it was important to verify the structure of the modified Tyr123 species in its functional, radical state. We performed a series of Q-band ¹H ENDOR measurements on activated Sp β prepared with various Tyr isotopologs (Fig. 4). We detected six distinct ¹H hyperfine interactions and, for all but one, determined the associated coupling constants. Representative spectra measured at the field position corresponding to the maximum EPR absorption of the radical are shown in Fig. 4; the complete set of orientation-selected ENDOR and high-field EPR measurements, along with associated analysis, are also provided (SI Appendix, Figs. S11–S14).

Fig. 2. Chemical and spectroscopic analysis of the radical oxidant in the class Ie Sp RNR β. (A) UV-visible absorption spectrum. (B) EPR spectra of Sp β (blue) and global simulations thereof (red), with natural-abundance Tyr• (Top) and β[2H₃] Tyr• (Bottom). (C) Spectrophotometrically monitored reductive titration by dithionite. (D) Loss of the radical oxidant and production of the nitrogen-centered radical (β•, black spectrum) on treatment of the holoenzyme with N3-CDP. (Insets) The double integral of the ν-N• dCDP spectra, accounting for >98% of the total spin. Procedures and sample compositions are described in SI Appendix.
The most prominent pair of peaks in the $^1$H ENDOR spectra corresponds to a coupling of approximately 30 MHz (SI Appendix, Table S6). This interaction is responsible for the doublet splitting that dominates the continuous-wave EPR spectrum. A sample prepared with $^{2}$H$_2$-Tyr did not exhibit the signal for this most strongly coupled $^1$H, confirming that this pair of ENDOR peaks arises from one of the two $^1$H atoms on C$_6$. We did not observe a second strong coupling from the other C$_6$ hydrogen; we estimate that it must be weaker than 2 MHz to have eluded detection. The strength of coupling for the C$_6$ hydrogens is proportional to the sine of the dihedral angle between the plane of the phenyl ring and the corresponding C$_6$-H bond (33). The results thus imply that one of the two C$_6$-H bonds lies nearly parallel to the phenyl plane, consistent with the orientation of the DOPA seen in the Au β structure ([phenyl]C$_6$-C$_4$ angle of ~60°–70°).

Spectra of a sample made with $[^2]$H$_2$-Tyr and in $^2$H$_2$O lack one pair of peaks present in the equivalent sample in $^2$H$_2$O, indicating that these peaks arise from an exchangeable hydrogen on the radical (see below). Two remaining signals from a pair of anisotropically coupled $^1$H nuclei are absent from spectra of a sample prepared with phenyl-$[^1]$H$_2$-Tyr (in either $^1$H$_2$O or $^2$H$_2$O), associating these signals with two ring hydrogens (Fig. 4 B and C). The weak-coupling region of the spectra reveals at least one more $^1$H coupling from the aromatic side chain; however, we could not confidently determine its hyperfine constant, owing to interference from weakly coupled, nonexchangeable $^1$H atoms of the protein matrix. A pair of ENDOR signals identified in all studied samples was unaffected by the isotopolog of Tyr or solvent and accounted for in simulations (see below) by a dipolar coupling of approximately 1.0–1.5 MHz. This observation implies the presence of at least one hydrogen nucleus at 3.7–4.2 Å from the center of spin density of the radical (according to the point-dipole approximation), likely a –CH$_3$ unit from a neighboring hydrophobic amino acid.

The hyperfine interaction from the exchangeable $^1$H, most obvious in the spectra of the phenyl-$[^2]$H$_2$-Tyr samples (Fig. 4 B and C), has a substantial isotropic component (~5 MHz; SI Appendix, Table S6), indicating that it is bonded to an atom harboring significant spin density. This signal, absent from corresponding spectra of known Tyr$^*$, can be assigned to the $^1$H on the C$_3$–OH of the neutral DOPA$^*$. The retention of this $^1$H can be rationalized based on the active-site H-bonding interactions seen in the crystal structure (see below) and is almost certainly essential for the tuning of the DOPA$^*$ for α-Cys oxidation.

The parameters used in a global simulation of all the ENDOR and continuous-wave EPR spectra (SI Appendix, Table S6) are consistent with the assignment of the radical as neutral DOPA$^*$. This conclusion is further supported by the consistency of the couplings with those from density functional theory calculations of a model of the radical (SI Appendix, Tables S6 and S7). The combined structural data thus imply that class Ie RNRs initiate catalysis with a DOPA$^*$, which is generated by a three-electron oxidation of a Tyr residue mediated by the flavoprotein NrdI.

Deep Mutational Scanning of Au β Reveals Sites Essential for DOPA Installation and Function. The distinctive modus operandi of the new Ie subclass upends expectations of the roles of conserved (and subclass e-specific) residues in activation and radical initiation. To assess the functional importance of selected residues, we leveraged the aforementioned Ec strain with the temperature-sensitive class Ia RNR α (13) as a selection for a deep mutational scanning (DMS) experiment (SI Appendix, Fig. S15) (34). We selected 19 residues in Au β (SI Appendix, Fig. S16), constructed a library of 32 genetic variants (encoding all 20 amino acids plus a...
residues are shown as ball-and-stick models and spheres, respectively, and color-coded by atom type and degree of substitution tolerance (dark green, intolerant to substitution; light green, moderately tolerant to conservative substitutions). (b) Amino acid enrichment/depletion map of inferred mutational tolerance of targeted residues. Red boxes (positive scores) indicate mutations highly enriched by selective growth at 42 °C; blue boxes (negative scores), mutations highly depleted by selective growth; gray boxes, mutations not tested due to incomplete coverage during library construction. Ovals indicate the wild-type residues at each site. One-letter amino acid codes are used. The stop codon TAG is indicated by an asterisk (*).

To assess the basis for the redox tuning of the DOPA* that is intolerant to substitution. Selected side chains and water molecules are shown as ball-and-stick models and spheres, respectively, and color-coded by atom type and degree of substitution tolerance (dark green, intolerant to substitution; light green, moderately tolerant to conservative substitutions). (b) Amino acid enrichment/depletion map of inferred mutational tolerance of targeted residues. Red boxes (positive scores) indicate mutations highly enriched by selective growth at 42 °C; blue boxes (negative scores), mutations highly depleted by selective growth; gray boxes, mutations not tested due to incomplete coverage during library construction. Ovals indicate the wild-type residues at each site. One-letter amino acid codes are used. The stop codon TAG is indicated by an asterisk (*).

A

B

Fig. 5. Results of DMS of Au β at 19 selected sites. (A) Sites near the DOPA* that are intolerant to substitution. Selected side chains and water molecules are shown as ball-and-stick models and spheres, respectively, and color-coded by atom type and degree of substitution tolerance (dark green, intolerant to substitution; light green, moderately tolerant to conservative substitutions). (b) Amino acid enrichment/depletion map of inferred mutational tolerance of targeted residues. Red boxes (positive scores) indicate mutations highly enriched by selective growth at 42 °C; blue boxes (negative scores), mutations highly depleted by selective growth; gray boxes, mutations not tested due to incomplete coverage during library construction. Ovals indicate the wild-type residues at each site. One-letter amino acid codes are used. The stop codon TAG is indicated by an asterisk (*).

Fig. 6. (A and B) Solvent access channels in β and β-NrdI interfaces in the class Ie Au and class Ib Ec RNR systems. Channels in Au β (A) and Ec class Ib β (PDB ID code 3N37) (B). (C and D) NrdI-β interfaces proposed for the Au complex from the structural model constructed in this study (C) and observed in the structure of Ec Ib β-NrdI (PDB ID code 3N3A) (D).

in the DMS experiment (Fig. 5 and SI Appendix, Fig. S16), form a water-mediated H-bonding network between Lys210 and the protein surface (SI Appendix, Fig. S17), and they likely play roles in controlling proton transfer(s) coupled to the α-Cys-to-DOPA* electron transfer that initiates turnover (8). The radical translocation process in class Ia RNRs involves the transfer of a proton from a water molecule coordinated to the Fe(III) ion in site 1 to the oxygen of the Tyr* (35). The lack of a transition metal in the active state of the class Ie RNR necessitates a different proton donor and pathway if, as expected, reduction of the DOPA* is coupled to proton transfer to the C4 O-atom.

The inferred transformation of the class Ie β protein by its NrdI activase—formally, hydroxylation and removal of an additional electron from a Tyr residue—is quite different from that effected in the class Ib system (Mn2⁺-iδO oxidation on the path to Tyr*) (4, 36), raising the possibility that the two NrdIIs might have different modes of action. However, the structure of the Au NrdIα (1.95 Å resolution) (SI Appendix, Fig. S18) shares with previously characterized Ib NrdI proteins (especially the Ec ortholog) (28) several functionally important features (SI Appendix, Figs. S18–S20). For example, a Gly-rich FMN-binding loop (50s loop) in Au NrdI donates a backbone N-H to H-bond with N5 of the flavin, an interaction that significantly occludes the O2-reactive C4a position (SI Appendix, Fig. S18). In Ec NrdI, the 50s loop opens on FMN reduction, and the C4a position becomes more exposed. The redox-dependent change controls reaction of the flavin with oxygen/superoxide (28, 36). Conservation of the loop in Au NrdI weighs in favor of O2 reduction to superoxide or peroxide by the class Ie activase. Moreover, a model for the Au β-NrdI complex, which we generated using the structure of the corresponding Ib Ec complex [Protein Data Bank (PDB) ID code 3N3A] (28) as a template, shows that the Ie β protein shares two defining structural adaptations of the Ib βs that enable (i) their complexation with NrdI and (ii) guidance of the reactive, hydrophilic oxidant (superoxide) to the buried cofactor site. The model is consistent with use of identical protein–protein interfaces in the Ib and Ie β-activase complexes; residues predicted to interact at the interface are highly conserved (Fig. 6 A and B). The second feature, a chain of hydrogen-bonded water molecules in Ie β lining the proposed pathway for superoxide migration, is also retained, interrupted only by the side chain of the Pro that replaces a Glu metal ligand in Ie proteins (Fig. 5 C and D). In addition, a strictly conserved surface Lys residue in Ib β (K260) (Fig. 6 D), a key

stop codon) for each site, and assembled them into the complementation plasmid. After transformation of the selection strain, KK444, amplicon libraries from preselection (28 °C) and post-selection (42 °C) populations were pooled and sequenced on the Illumina HiSeq platform. No functionally competent substitution was identified (Fig. S16) in this experiment.

To assess the basis for the redox tuning of the DOPA* that is intolerant to substitution in α, we included the adjacent residues, Asp85 and Lys210, in the DMS assay. Neither residue can be substituted with any other natural amino acid present in the initial library without loss of function. As judged by the ability to complement the temperature-sensitive mutant strain at the restrictive temperature, inspection of the X-ray structure suggests that one of the Asp85 side-chain carboxylate O-atoms makes a short H-bond with the newly installed O-atom of DOPA123 (SI Appendix, Fig. S17). Comparison of the positioning of DOPA relative to Asp85 in each of the four chains of the asymmetric unit in the activated Au β crystals (Fig. 3A and SI Appendix, Fig. S7) reveals two distinct states, I and II, of the DOPA/Asp85 dyad. One of these states could represent an intermediate, as in a form that undergoes hydroxylation but not further oxidation to the radical, in a two-step activation process. Alternatively, failed activation events or adventitious reduction to the inactive DOPA state in a fraction of the monomers could account for the differences. In all structures, the other Asp85 side-chain oxygen H-bonds to the side chain of Lys210. These interactions would maintain both the DOPA* and the Lys210 nitrogen in their protonated forms (SI Appendix, Fig. S17). In general, a proximal positive charge should elevate the reduction potential of the DOPA*. Residues His119 and Trp49, also intolerant to every substitution sampled.

Fig. 5. Results of DMS of Au β at 19 selected sites. (A) Sites near the DOPA* that are intolerant to substitution. Selected side chains and water molecules are shown as ball-and-stick models and spheres, respectively, and color-coded by atom type and degree of substitution tolerance (dark green, intolerant to substitution; light green, moderately tolerant to conservative substitutions). (b) Amino acid enrichment/depletion map of inferred mutational tolerance of targeted residues. Red boxes (positive scores) indicate mutations highly enriched by selective growth at 42 °C; blue boxes (negative scores), mutations highly depleted by selective growth; gray boxes, mutations not tested due to incomplete coverage during library construction. Ovals indicate the wild-type residues at each site. One-letter amino acid codes are used. The stop codon TAG is indicated by an asterisk (*).

Fig. 6. (A and B) Solvent access channels in β and β-NrdI interfaces in the class Ie Au and class Ib Ec RNR systems. Channels in Au β (A) and Ec class Ib β (PDB ID code 3N37) (B). (C and D) NrdI-β interfaces proposed for the Au complex from the structural model constructed in this study (C) and observed in the structure of Ec Ib β-NrdI (PDB ID code 3N3A) (D).

10026 | www.pnas.org/cgi/doi/10.1073/pnas.1811993115
Blaesi et al.
component of the NrdD interface proposed to guide the anionic superoxide to the Mn$_2^{III}$ cluster, is also present in the Ie β. These observations are all consistent with similar roles for the Ie and Ib NrdD proteins.

Important aspects of the mechanism of activation of the new class Ie β remain to be resolved. For example, it is not clear whether the three-electron oxidation of Tyr123 to DOPA• requires a transition metal—as in the posttranslational installation of most known quinocorators (37)—that subsequently dissociates or requires no metal assistance at all. In the former case, the transient metal requirement would be reminiscent of the activation mechanism of a class III RNR [iron-dependent installation of a catalytic Gly in a metal-free RNR (7)] and would render the class Ie enzymes the aerobic counterpart of this catalytic metal usage. Similarly, the identity and stoichiometry of the (presumably) O$_2$-derived oxidant(s) are not known, although the structural analogy to the Ib systems and the fact that DOPA• production is a three-electron oxidation make the use of a single equivalent of superoxide the simplest possibility. Interestingly, a structure of Au β solved with thiocyanate as additive reveals the iron bound noncovalently in a pocket immediately adjacent to the Lys210-Asp85-Tyr123 triad (SI Appendix, Fig. S21). The similarity of thiocyanate to superoxide in size, structure, and charge raises the possibility that this pocket binds the oxidant before its reaction with Tyr123 or a transiently bound metal ion.

Methods

Detailed information on metal analysis, spectroscopy, X-ray crystallography, mass spectrometry, and mutational scanning is provided in SI Appendix. A summary is provided here.