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Bacillus subtilis Class Ib Ribonucleotide Reductase: High Activity and Dynamic Subunit Interactions

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Supporting Information

ABSTRACT: The class Ib ribonucleotide reductase (RNR) isolated from Bacillus subtilis was recently purified as a 1:1 ratio of NrdE (α) and NrdF (β) subunits and determined to have a dimanganese-tyrosyl radical (∈MnIII-Y) cofactor. The activity of this RNR and the one reconstituted from recombinantly expressed NrdE and reconstituted MnIII-Y·NrdF using dithiothreitol as the reductant, however, was low (160 nmol min⁻¹ mg⁻¹). The apparent tight affinity between the two subunits, distinct from all class Ia RNRs, suggested that B. subtilis RNR might be the protein that yields to the elusive X-ray crystallographic characterisation of an “active” RNR complex. We now report our efforts to optimize the activity of B. subtilis RNR by (1) isolation of NrdF with a homogeneous cofactor, and (2) identification and purification of the endogenous reductant(s). Goal one was achieved using anion exchange chromatography to separate apo-/mismetalated-NrdFs from MnIII-Y·NrdF, yielding enzyme containing 4 Mn and 1 Y/β. Goal two was achieved by cloning, expressing, and purifying TrxA (thioredoxin), YosR (a glutaredoxin-like thioredoxin), and TrxB (thioredoxin reductase). The success of both goals increased the specific activity to ~1250 nmol min⁻¹ mg⁻¹ using a 1:1 mixture of NrdE:MnIII-Y·NrdF and either TrxA or YosR and TrxB. The quaternary structures of NrdE, NrdF, and NrdE:NrdF (1:1) were characterized by size exclusion chromatography and analytical ultracentrifugation. At physiological concentrations (~1 μM), NrdE is a monomer (α) and MnIII-Y·NrdF is a dimer (β). A 1:1 mixture of NrdE:NrdF, however, is composed of a complex mixture of structures in contrast to expectations.

Ribonucleotide reductases (RNRs, Figure 1) catalyze the conversion of nucleoside 5′-diphosphates (NDPs) to deoxynucleotides (dNDPs) and play an essential role in supplying balanced dNTP pools for DNA replication and repair. The RNRs have been classified based on the metallocofactors used to generate a transient cysteine thiyl radical that is essential for catalysis. The class I RNRs contain two subunits (α and β) with subclassification (Ia, Ib, and Ic, Figure 1) based on the dimetallocofactor located in β. Class Ib RNRs, as with class Ia enzymes, were long thought to use a diferric-tyrosyl radical (FeIII-Y) cofactor because the cluster can self-assemble and has catalytic activity. Recently, however, the class Ib RNRs have been isolated and characterized from their endogenous sources (Corynebacterium ammoniagenes, Escherichia coli, and Bacillus subtilis) in sufficient amounts to establish that the cofactor is a dimanganese-tyrosyl radical (∈MnIII-Y). While most prokaryotes have multiple RNRs, B. subtilis has a single essential class Ib RNR found in the nrdl:nrdE:nrdF:ymaB operon. The apparent tight binding of the subunits of the B. subtilis RNR and the low reported specific activities of this and other class Ib enzymes (Supporting Information, SI-Table 1), provided the impetus to maximize its catalytic activity for future structural determination.

In all class I RNRs, α (designated NrdE for class Ib and NrdA for class Ia, Figure 1) contains the active site for NDP reduction and binding sites for allosteric effectors that control substrate specificity. The class Ia α contains a second allostERIC site in its N-terminal ATP cone domain that controls the overall rate of nucleotide reduction and is absent in the class Ib α subunits. The β subunit (NrdF for class Ib and NrdB for class Ia) is all dimeric (β) with the class Ia housing a FeIII-Y·cofactor (typically with 3.6 Fe/β and 1 Y/β) and the class Ib housing a MnIII-Y·β, whose stoichiometry remains to be optimized in all systems.

Recent data suggest that in addition to allosteric effectors, quaternary structure(s) of the class I RNRs, including subunit affinity and its dependence on nucleotide binding, are all important in regulating RNR activity. The subunit affinity in the E. coli and human class Ia RNRs have reported Kᵦ's of ~0.2 μM in the presence of substrate (CDP)/effector (ATP). While there is now a consensus that the active quaternary structure of E. coli class Ia RNR is αβₜ, the eukaryotic Ia structure is open to debate [(α₁β₁)(β₂)ₙ (n = 1, 3 and m = 1, 3)]. Recent studies have further revealed that the active complexes of both the prokaryotic and eukaryotic RNRs are dynamic. The quaternary structures of the class Ib RNR might be the protein that yields to the elusive X-ray crystallographic characterisation of an “active” RNR complex. We now report our efforts to optimize the activity of B. subtilis RNR by (1) isolation of NrdF with a homogeneous cofactor, and (2) identification and purification of the endogenous reductant(s). Goal one was achieved using anion exchange chromatography to separate apo-/mismetalated-NrdFs from MnIII-Y·NrdF, yielding enzyme containing 4 Mn and 1 Y/β. Goal two was achieved by cloning, expressing, and purifying TrxA (thioredoxin), YosR (a glutaredoxin-like thioredoxin), and TrxB (thioredoxin reductase). The success of both goals increased the specific activity to ~1250 nmol min⁻¹ mg⁻¹ using a 1:1 mixture of NrdE:MnIII-Y·NrdF and either TrxA or YosR and TrxB. The quaternary structures of NrdE, NrdF, and NrdE:NrdF (1:1) were characterized by size exclusion chromatography and analytical ultracentrifugation. At physiological concentrations (~1 μM), NrdE is a monomer (α) and MnIII-Y·NrdF is a dimer (β). A 1:1 mixture of NrdE:NrdF, however, is composed of a complex mixture of structures in contrast to expectations.

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RNRs, especially with the Mn\textsuperscript{III}-Y cofactor, have been much less extensively studied and remain to be determined.\textsuperscript{3,23,24} Since the establishment that class Ib RNRs possess a Mn\textsuperscript{III}-Y cofactor, many efforts have been made to isolate and characterize these enzymes.\textsuperscript{5–9,23–25} With one exception\textsuperscript{4}, the reported activities of purified class Ib RNRs, whether endogenously or recombinantly sourced, are substantially lower (Supporting Information, SI-Table 1) than their class Ia counterparts (5000–8000 nmol min\textsuperscript{−1} mg\textsuperscript{−1}).\textsuperscript{26} Recently our lab isolated the class Ib RNR from \textit{B. subtilis} with NrdE and NrdF copurifying in a 1:1 ratio through three chromatographic steps in the absence of nucleotides and Mg\textsuperscript{2+}, suggesting “tight” subunit affinity.\textsuperscript{4} However, while the RNR was 80% pure, its catalytic activity was low (∼160 nmol min\textsuperscript{−1} mg\textsuperscript{−1}). Efforts to reconstitute active RNR using recombinantly expressed NrdE and reconstituted Mn\textsuperscript{III}-Y NrdF also resulted in low catalytic activity.\textsuperscript{9,27}

In this paper, we report optimization of the catalytic activity of the \textit{B. subtilis} RNR by separation of active Mn\textsuperscript{III}-Y loaded-NrdF (Me = Fe or Mn) from the apo- and mismetalated forms and that the active form has 1 Y\textsubscript{NrdF} (Me = Fe or Mn) from the apo- and mismetalated forms revealed that the use of the endogenous reductants contributed significantly more to the activity enhancement relative to the cluster loading of NrdF. Activity assays with CDP/ATP and increasing concentrations of NrdE:NrdF maintained in a 1:1 ratio gave an apparent \( K_m \) of 25 mM, 10-fold lower than the apparent \( K_m \) for the Ia RNRs with the same substrate/effecter pair.\textsuperscript{13,14} The quaternary structure of each subunit and a 1:1 mixture of NrdE:NrdF at physiological concentrations (1 \( \mu \text{M} \)) was examined by size exclusion chromatography (SEC) and sedimentation velocity analytical ultracentrifugation (SV-AUC) in the absence of nucleotides. These studies reveal that NrdE is a monomer, Me\textsuperscript{III}-Y NrdF is a dimer, and the 1:1 mixture of NrdE and NrdF is a mixture of subunits and higher molecular weight species. These studies set the stage for determination of nucleotide binding and how it effects subunit interactions and quaternary structure in a class Ib RNR.

### MATERIALS AND METHODS

#### Materials

Chemical reagents were obtained from Sigma-Aldrich at the highest purity available. Restriction enzymes, T4 DNA ligase, and isopropyl-β-D-1-thiogalactoside (IPTG) were purchased from Promega (Madison, WI). All DNA sequencing was performed at the Massachusetts Institute of Technology Biopolymers Laboratory. Ni-NTA Fast Flow affinity resin was from Qiagen. Complete EDTA-free protease inhibitor tablets and calf alkaline phosphatase (20 \( \mu \text{mol min}^{-1} \mu\text{L}^{-1} \)) were purchased from Roche Biochemicals (Indianapolis, IN). Amicon Ultra-15 centrifugal filter devices were from Millipore. N-Terminally His\textsubscript{tagged} NrdE and apo-NrdF were expressed and purified as reported previously.\textsuperscript{9,27} Apo-NrdE was reconstituted with Mn\textsuperscript{II}-Y and Fe\textsuperscript{III}-Y as previously described.\textsuperscript{9,28}

#### Cloning, Expression, and Purification of N-Terminally His\textsubscript{tag}ed TrxA, TrxB, and YosR

The genes \textit{trxA}, \textit{yosR}, and \textit{trxB} were amplified by PCR using \textit{B. subtilis} JJ624 genomic DNA (a gift from A. Grossman in the Department of Biology, Massachusetts Institute of Technology) as a template and the primers listed in Supporting Information, SI-Table 2. The amplified DNA fragments were digested with NdeI and XhoI and ligated into pET14b (Novagen) using T4 DNA ligase to create the vectors pXZtrxA, pXZtrxB, and pXZyosR.

TrxA and YosR were overproduced from pXZtrxA and pXZyosR, respectively, transformed into BL21 (DE3) \textit{pLysS E. coli} cells (Invitrogen). Cells were grown in 1 L of ampicillin-supplemented (Amp, 100 \( \mu\text{g/mL} \)) LB broth in 6 L Erlenmeyer flasks at 37 °C with 200 rpm shaking. At an OD\textsubscript{600} of 0.8, IPTG was added to a final concentration of 0.2 mM and cultures were incubated for an additional 2 h before harvest. TrxB was overproduced from pXZtrxB transformed into BL21 (DE3) \textit{pLysS E. coli} cells (Agilent Technologies) following the same procedure with the exception that baffled 2.8 L Fernbach flasks were used for culture growth and incubation postinduction was extended to 3 h before harvest. Typical yields were 2.4–3.0 g cell paste L\textsuperscript{−1} for all strains.

The purification of all three proteins was the same through the nickel affinity step unless otherwise noted and was carried out at 4 °C. Cell pellets were thawed on ice and resuspended to 0.2 g paste mL\textsuperscript{−1} in buffer A (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 5% (w/v) glycerol, pH 7.6) supplemented with protease inhibitors. Cells were lysed by passage through a French pressure cell at 14 000 psi and the debris was removed by centrifugation (35000g, 20 min). Streptomycin sulfate solution was added to the stirring cell extracts dropwise over 10 min to a final concentration of 1% (w/v). After an additional 15 min of stirring, precipitated proteins were collected by centrifugation and resuspended in buffer A. All purification steps were performed at 4 °C.
materials were removed by centrifugation (35000g, 20 min). The streptomycin treatment was not used in the purification of TrxB as it caused precipitation of the desired protein. Clarified extracts were incubated with Ni-NTA resin equilibrated in buffer A for 1 h (1 mL of resin per 2.5 g of cell paste) on a rocker. The resin was then packed into a column and washed with 20 column volumes (CVs) of buffer B (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, 5% (w/v) glycerol, pH 7.6) before eluting protein with buffer C (50 mM sodium phosphate, 300 mM NaCl, 200 mM imidazole, 5% (w/v) glycerol, pH 7.6). Protein-containing fractions were identified using 15% SDS-PAGE gels and were then pooled and desalted on a Sephadex G-25 column with buffer D (50 mM sodium phosphate, 5% (w/v) glycerol, pH 7.6).

TrxA was purified further with a Sephadex G-75 column (41 × 2.5 cm, 0.25 mL min⁻¹) equilibrated with SEC buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.6). The fractions containing TrxA, assessed by SDS-PAGE, were pooled and desalted with an Amicon Ultra-15 YM-3 centrifugal filter (3000 MWCO). Typical yields were 11 ± 4 mg TrxA g⁻¹ cell paste. Purity was assessed by 15% SDS-PAGE (Supporting Information, SI-Figure 1), and protein concentrations were determined as previously described.32 SAs of YosR and TrxB of ∼8, 26 U/mg and 415 U/mg, respectively, were determined using the BCA assay.29,30

Subsequent to Cofactor Assembly. 7.6) supplemented with 100 mM NaCl. The column was washed with 1 CV of equilibration buffer before development with a 100 mL linear gradient from 100 to 700 mM NaCl in AEX buffer. YosR eluted in the range of 350–450 mM NaCl (flow rate = 2 mL min⁻¹) and TrxB in the range of 230–280 mM NaCl (flow rate = 1 mL min⁻¹). Fractions containing TrxB were pooled by activity, exchanged into 50 mM NaPi, pH 7.6, 150 mM NaCl, and concentrated with an Amicon Ultra-15 YM30 centrifugal filter device (30000 MWCO). Fractions containing YosR, as assessed by 15% SDS-PAGE, were pooled, desalted, and concentrated with an Amicon Ultra-15 YM-3 centrifugal filter device. YosR was subjected to further purification using the SEC method described for TrxA. Typical yields were 3 mg YosR g⁻¹ cell paste and 0.4 mg TrxB g⁻¹ cell paste. Purity was assessed by 15% SDS-PAGE (Supporting Information, SI-Figure 1). The concentration of YosR was estimated by ε₂⁸⁰ of 12 700 M⁻¹ cm⁻¹ (DNASTAR Lasergene 8). The specific activity (SA) of the purified TrxA was ~8–20 U/mg by the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay.29,30

YosR and TrxB were further purified by anion exchange chromatography on a BioCAD SPRINT FPLC system (PerSeptive Biosystems). Protein was loaded onto a Poros HQ/20 column (Applied Biosystems, 1.6 cm × 10 cm) equilibrated in AEX buffer (25 mM Tris, 5% (v/v) glycerol, pH 7.6) supplemented with 100 mM NaCl. The column was washed with 1 CV of equilibration buffer before development with a 100 mL linear gradient from 100 to 700 mM NaCl in AEX buffer. TrxA eluted in the range of 350–450 mM NaCl (flow rate = 2 mL min⁻¹) and TrxB in the range of 230–280 mM NaCl (flow rate = 1 mL min⁻¹). Fractions containing TrxB were pooled by activity, exchanged into 50 mM NaPi, pH 7.6, 150 mM NaCl, and concentrated with an Amicon Ultra-15 YM30 centrifugal filter device (30000 MWCO). Fractions containing YosR, as assessed by 15% SDS-PAGE, were pooled, desalted, and concentrated with an Amicon Ultra-15 YM-3 centrifugal filter device. YosR was subjected to further purification using the SEC method described for TrxA. Typical yields were 3 mg YosR g⁻¹ cell paste and 0.4 mg TrxB g⁻¹ cell paste. Purity was assessed by 15% SDS-PAGE (Supporting Information, SI-Figure 1). The concentration of YosR was estimated by ε₂⁸⁰ of 11 000 M⁻¹ cm⁻¹ (DNASTAR Lasergene 8). The concentration of TrxB was estimated with the BCA assay using BSA as a standard.32 The FAD content of TrxB was determined as previously described.32 SAs of YosR and TrxB of ~20–26 U/mg and 415 U/mg, respectively, were determined using the DTNB assay.29,30 One unit is defined as the amount of enzyme catalyzing a ΔA₄₅₀ of 1.0 min⁻¹.32,30

**Purification of NrdE.** NrdE was purified as previously described except that buffer supplementation with NaCl and an additional anion exchange chromatographic step were introduced (see Supporting Information).9

**Purification of holo-Me³⁺Ⅲ⁻Ⅲ⁻Y.** (Me = Fe or Mn) Subsequent to Cofactor Assembly. EDTA (pH 7.6) to a final concentration of 5 mM was added to 1 mL of reconstituted Me³⁺Ⅲ⁻Y. NrdF (40 μM Mn-loaded protein or 300 μM Fe-loaded protein, ~0.6 Y⁻/β⁻). Subsequent steps were at 4 °C. Samples were mixed, clarified by centrifugation (20817g, 2 min), and loaded onto a MonoQ 10/100GL anion exchange column (GE Healthcare, 10 × 1 cm) equilibrated with 50 mM Tris, pH 7.6, 5% (w/v) glycerol and 100 mM NaCl. The column was washed with 1.3 CVs of equilibration buffer before elution with a 100 mL linear gradient from 100 to 350 mM NaCl in Tris buffer at a flow rate of 1 mL min⁻¹. Apo-/mis-metalated NrdF eluted at 250–280 mM NaCl (peak 1, Figure 2) and holo-protein at 280–310 mM (peak 2, Figure
mM HEPES, pH 7.6, 15 mM MgCl\(_2\) and 1 mM EDTA at 37 °C. The reaction was initiated by addition of [\(^{3}H\)]-CDP. Aliquots (35 µL) were removed over 4 min and quenched by incubation at 100 °C for 2 min. The method of Steeper and Steuart was used to quantitate dCDP.\(^{35}\) One unit of activity is the amount of enzyme required to catalyze 1 nmol dCDP min\(^{-1}\). The effect of the allosteric effectors dATP (0.4 µM to 4 mM) or ATP (50 µM to 4 mM) on activity was carried out as described above.

The concentration dependence of RNR activity using a 1:1 mixture of α and β was measured with subunit ratios varying from 0.01–1 µM. At concentrations below 0.1 µM, BSA was included in the assay solution to a final concentration of 0.2 mg/mL. These data were fit to eq 1 with Igor Pro (WaveMetrics, Lake Oswego, OR).

\[
\nu = \frac{V_{\text{max}}[\text{subunit}]}{K_m + [\text{subunit}]}
\]

### Quaternary Structural Analysis of 1:1 Mixtures of αβ Using Biophysical Methods.

#### A. Anion Exchange Chromatography. NrdE and Me\(^{3+}\)-Y. NrdF (Me = Fe or Mn, 1 Y/β, \~3.6 Me\(^{3+}\)/β) were mixed in a 1:1 ratio at 5 µM in 50 mM HEPES, 5% (w/v) glycerol, pH 7.6 in a total volume of 0.5–1 mL. Samples were incubated on ice for 20 min and at room temperature for 10 min, followed by centrifugation (20817g, 1 min, 4 °C). All subsequent steps were at 4 °C. Samples were injected onto a MonoQ anion exchange column equilibrated in 50 mM Tris, pH 7.6, 5% (w/v) glycerol and 100 mM NaCl. The column was washed with 1 CV of the equilibration buffer and then developed with a 160 mL linear gradient from 100 to 500 mM NaCl in Tris buffer at a flow rate of 1 mL min\(^{-1}\) (Supporting Information, SI-Figure 2). Fractions (2 mL) containing protein were identified by A\(_{280}\) pooled, exchanged into storage buffer (50 mM HEPES, pH 7.6, 5% (w/v) glycerol), and concentrated using an Amicon Ultra-15 YM30 centrifugal filter. The metal content, radical content, and activity of the isolated complex (50 mM HEPES, pH 7.6, 5% (w/v) glycerol, 150 mM NaCl, 15 mM MgCl\(_2\), 1 mM DTT) was measured with subunit ratios varying from 0.01 to 1 µM.

#### B. SEC. Samples of NrdE (3 µM), holo-Fe\(^{3+}\)-Y. NrdF (23 µM), holo-Mn\(^{3+}\)-Y. NrdF (1 µM), and 1:1 mixtures of αβ (1 µM) in a total volume of 200 µL were centrifuged (20817g, 10 min, 4 °C) and then injected onto a Superdex 200 10/300 GL column (10 × 300 mm, ~24 mL) connected to an ÄKTA Purifier FPLC system (GE Healthcare). Protein was eluted at 4 °C at a flow rate of 0.25 mL min\(^{-1}\) using 50 mM HEPES, pH 7.6, 100 mM NaCl, 15 mM MgCl\(_2\), 1 mM EDTA. Molecular weights were calculated as previously described\(^{36}\) using the experimentally measured s\(_{20,w}\) (subsequent session), the Stokes radius (R\(_{s}\)) estimated from the SEC retention times, and the correlation function of Laurent and Killander.\(^{37}\) The column was calibrated using a High Molecular Weight Gel Filtration kit (GE Healthcare) containing the following standards: Blue Dextran 2000 (void volume determination), thyroglobulin (669 kDa, R\(_{s}\) = 85.0 Å), ferritin (440 kDa, R\(_{s}\) = 61.0 Å), aldolase (158 kDa, R\(_{s}\) = 48.1 Å), conalbumin (75 kDa, R\(_{s}\) = 36.4 Å\(^{38}\)), and ovalbumin (44 kDa, R\(_{s}\) = 30.5 Å).

#### C. SV-AUC—Experimental Design. Concentration-dependent SV experiments were performed using a Beckman XL-I analytical ultracentrifuge at the MIT Biophysical Instrumentation Facility with Fe\(^{3+}\)/β (1.2 Y/β, 4.0 Fe/β, 130 U/mg), Mn\(^{3+}\)/β (0.9 Y/β, 4.0 Mn/β, 1545 U/mg), and α. Individual subunits were analyzed in 50 mM Tris, pH 7.6, 5% (w/v) glycerol, 150 mM NaCl, 15 mM MgCl\(_2\), 1 mM DTT, whereas 1:1 mixtures of α and β were analyzed in 50 mM NaPi, pH 7.6, 150 mM NaCl, 5% (w/v) glycerol, 1 mM TCEP to allow experiments to be monitored by A\(_{280}\). Protein was exchanged into buffer using three cycles of concentration-dilution with an Amicon Ultra-15 YM30 centrifugal filter. The final filtrate was used as the SV reference buffer. Samples spanning 0.1–1.5 OD at A\(_{280}\), A\(_{350}\), or A\(_{280}\) (Supporting Information, SI-Table 3) were prepared in a total volume of 500 µL by mixing concentrated protein solution with reference buffer to the desired final concentration for each experiment. Epon charcoal double sector cells (12 mm) assembled with quartz windows were loaded with ~440 µL reference solution and ~430 µL sample solution, radially calibrated, and thermally equilibrated at 20 °C for 1.5–2 h prior to initiating the experiment. Sedimentation was measured over 18–19 h with absorption detection at 20 °C and an angular velocity of 35 000 rpm. Scans were collected every 1.2 min using ProteomeLab XL-I Graphical User Interface, version 4.5b (Beckman).\(^{39}\)

### C1. Analysis Method. Individual data sets were fit with the c(s) model implemented in Sedfit with a resolution (N) = 200–250, and a regularization factor (P) = 0.95.\(^{40,41}\) Distributions were converted to the standard state (s\(_{20,w}\)) using a solvent density (ρ) = 1.018 g mL\(^{-1}\) (Tris buffer) or 1.023 g mL\(^{-1}\) (NaPi buffer), solvent viscosity (η) = 1.164 cp (Tris buffer) or 1.170 cp (NaPi buffer), and the partial specific volume (v) of the species analyzed (Supporting Information, SI-Table 4); these values were calculated and temperature corrected with Sednterp.\(^{42}\) All Mn\(^{3+}\)-Y. NrdF data sets and the NrdE and NrdE:Fe\(^{3+}\)-Y. NrdF data collected at physiological concentrations (~1 µM) were fit with the program Sedphat using the “hybrid local continuous/global discrete species model” for a more rigorous determination of s\(_{20,w}\) and M\(_{w}\). The best fit s\(_{20,w}\) for each species was used to determine R\(_{s}\) and the frictional ratio (f/f\(_{ref}\)) with Sednterp by turning off the temperature corrections, setting ρ = 0.998 g mL\(^{-1}\) and η = 1.002 cp, and calculating the M\(_{w}\) values using the amino acid sequences of NrdE and NrdF including the His-tag.

#### D. Hydrodynamic Modeling. Predictions of the s\(_{20,w}\) frictional ratio (f/f\(_{ref}\)) and R\(_{s}\) for each species were determined using the program HYDROPRO\(^{43}\) and structural models of NrdE and NrdF (PDB 4DRO) (Supporting Information, SI-Table 4). A crystallographic structure of B. subtilis NrdE is not available, and therefore the web-based server PHYRE\(^{45}\) was used to generate a NrdE model by threading onto the Salmonella typhimurium NrdE structure (PDB 1PEM, 45% identity to B. subtilis NrdE; see Supporting Information).\(^{46}\)

#### SDS-PAGE Densitometry. The ratio of αβ in 1:1 mixtures was estimated by densitometry using standard curves prepared from known amounts of NrdE and NrdF as described previously.\(^{9}\) See Supporting Information for details.

#### RESULTS

**Purification of Me\(^{3+}\)-Y. NrdF (Me = Fe or Mn) to give 1 Y/β.** The low amounts of Y- and metal content in class β subunits examined to date suggest that NrdFs (Supporting Information, SI-Table 1) are heterogeneous mixtures of holoheme apo- and mismetalated protein. On the basis of the report of partial separation of Bacillus anthracis holo- and apo-NrdF,\(^{24}\) B. subtilis NrdF was examined on different anion exchange resins with MonoQ ultimately effecting complete separation of holoheme Feβ- and Mn-β from apo- and mismetalated-NrdF (Figure 2). Analysis of pooled peak 2 by atomic absorption and EPR
spectroscopy revealed 4.1 ± 0.3 Mn/β, and 1.00 ± 0.09 Y/β, for MnII,Y-NrdF and by ferrozine assay and EPR spectroscopy 4.0 ± 0.1 Fe/β, and 1.05 ± 0.05 Y/β, for FeII,Y-NrdF (Supporting Information, SI-Figure 3). In contrast, pooled protein from peak 1 (Figure 2) typically had 0.1 Y/β, ~1.0 metal/β, and no detectable activity. Holo-NrdF from peak 2 has been used in all subsequent experiments.

Purification of B. subtilis TrxA, YosR, and TrxB. The highest activities of class I RNRs have been achieved with endogenous reducing systems that are distinct for different class Ib RNRs (see Supporting Information, SI-Table 1).26,48 Mining of B. subtilis JH624 genome revealed a number of candidate genes. Thioredoxin (trxA, TR), YosR (yosR, a NrdH or glutaredoxin-like thioredoxin25,47), and thioredoxin reductase (trxB, TRR) were ultimately pursued due to homology to the reductants that maximally supported RNR activity in B. anthracis.25 Histagged versions of these genes were cloned, overexpressed in E. coli, and purified by Ni-affinity chromatography. Each protein required additional purification steps. For TrxA, Sephadex G-75 SEC yielded ≥99% pure protein (Supporting Information, SI-Figure 1) with a specific activity of ~11–20 U/mg using the DTNB assay in the presence of NADPH and TrxB. For YosR, Poros HQ/20 FPLC anion exchange chromatography followed by Sephadex G-75 SEC resulted in ≥95% pure protein (Supporting Information, SI-Figure 1) with a specific activity of ~20–26 U/mg. Finally, nearly homogeneous TrxB with a full complement of FAD was obtained with Poros HQ/20 anion exchange (≥95% pure, Supporting Information, SI-Figure 1) and had a specific activity of 415 U/mg in the presence of either TrxA or YosR.

Assay Optimization. Historically, the activity of each subunit for class Ia RNRs has been assayed independently in the presence of an excess of the second subunit due to weak subunit interactions.26,48 Similar assays of Ib RNRs have been carried out using this protocol.3,5,8,9,24,34,49 Our previous results, however, suggested the B. subtilis RNR could be assayed as a holo-enzyme by using equivalent amounts of each subunit.9 To validate the use of a 1:1 ratio of subunits, experiments were therefore initially carried out using 0.5–1 μM NrdF, increasing amounts of NrdE (1, 5, or 10 equiv), 3 mM ATP, and the endogenous redactants TrxA/TrxB/NADPH. The results with both Fe- and Mn-loaded NrdF in a 1:1 ratio with NrdE gave activity very similar to that in the presence of a 5–10-fold excess of NrdE (data not shown). Thus, all further studies used a 1:1 ratio of the subunits.

Optimization of Effector (ATP, dATP) Concentrations. Previous studies of the Fe-loaded S. typhimurium, Lactococcus lactis, and Mycobacterium tuberculosis class Ib RNRs reported that dATP strongly stimulated CDP reduction, while ATP had only a marginal stimulatory affect.3,5,49,50 These results contrast with the class Ia RNRs, in which dATP stimulates activity by binding to the specificity site of α at low concentrations and inhibits at high concentrations by binding to both the specificity and activity site leading to an αβ inhibitor complex.6,11 This quaternary structure is inaccessible in Ib RNRs as they lack the ATP cone domain essential for αβ inhibitor formation.22 Thus, studies with both ATP and dATP were carried out, and the results are shown in Figure 3. ATP maximally stimulated CDP reduction at 1.5–2 mM, while dATP showed maximal stimulation at 8–10 μM followed by rapid inhibition at higher concentrations. The effects were similar with Fe-loaded NrdF (data not shown), although the activity was 5-fold lower. The dATP behavior was unexpected as there is no ATP cone domain, and it is distinct from other reported class Ib enzymes.3,5,49,50 The Mg2+ dependence was also examined and gave an optimum activity at 10–15 mM, while EDTA had no effect on the activity (data not shown).

The SA of B. subtilis RNR under optimized conditions with CDP/(d)ATP is among the highest reported for any member of this subclass (Table 1; Supporting Information, SI-Table 1).

Figure 3. Effector nucleotide concentration-dependence of CDP reduction by a 1:1 mixture of MnII,Y-NrdF and NrdE. Assays were conducted with 0.5 μM of each subunit. Error bars represent ±1 standard deviation from the mean. (A) Dependence of B. subtilis RNR activity on ATP. (B) Dependence of B. subtilis RNR activity on dATP.

Table 1. Optimized CDP Reduction Activity of B. subtilis NrdFα

<table>
<thead>
<tr>
<th>Me</th>
<th>reductantβ</th>
<th>effector</th>
<th>SA (U/mg)</th>
</tr>
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<tbody>
<tr>
<td>Fe</td>
<td>DTT</td>
<td>3 mM ATP</td>
<td>52 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM ATP</td>
<td>22</td>
</tr>
<tr>
<td>TrxA</td>
<td>3 mM ATP</td>
<td>125 ± 23</td>
<td></td>
</tr>
<tr>
<td>YosR</td>
<td>3 mM ATP</td>
<td>106 ± 8</td>
<td></td>
</tr>
<tr>
<td>Mnc</td>
<td>DTT</td>
<td>3 mM ATP</td>
<td>146 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM ATP</td>
<td>160</td>
</tr>
<tr>
<td>YosR</td>
<td>3 mM ATP</td>
<td>1475 ± 129</td>
<td></td>
</tr>
<tr>
<td>TrxA</td>
<td>7 μM dATP</td>
<td>1257 ± 128</td>
<td></td>
</tr>
</tbody>
</table>

αPurified MnIII,Y-NrdF characteristics: Feβ2 = 1.1 Y/β; 3.6 Fe/β, Mn−β2 = 1.0 Y/β, 3.2 Mn/β. βDTT, final concentration 20 mM. TrxA or YosR, the final concentration 40 μM and assays included 0.4 μM TrxB and 1 mM NADPH. γPrevious study. δUnpurified FeII,Y-NrdF (0.5 Y/β, 28 Fe/β). εUnpurified MnII,Y-NrdF (0.6 Y/β, 2.6 Mn/β).
system had a much greater effect on activity (5–10-fold enhancement with Mn\(^{II}\)·Y-) than did cluster assembly (0–2-fold enhancement, Table 1). This starkly contrasts with the B. anthracis RNR, where similar activities were observed with DTT and Trx1.25

**Reconstitution of Active RNR from Recombinant NrdE and Reconstituted NrdF.** The most distinct aspect of the copurification of endogenous NrdE and NrdF was that the complex was maintained in the absence of substrates, effectors, and Mg\(^{2+}\).7 Analysis of a 1:1 mixture NrdE:NrdF (5 μM) on a MonoQ_FPLC column reproduced this result (Supporting Information, SI-Figure 2). A single peak eluted between 380–410 mM NaCl, behavior distinct from NrdE alone (two peaks that eluted between 265–300 mM NaCl and 325–340 mM NaCl) and reconstituted NrdF (two peaks that eluted in the range of 250–280 mM NaCl and 280–310 mM NaCl) using the same NaCl gradient. SDS-PAGE densitometry analysis of the fractions showed a 1:1 subunit ratio (Supporting Information, SI-Figure 4). Both the recovered Fe- and Mn-loaded complexes were active in CDP reduction (116 ± 5 U/mg and 889 ± 6 U/mg, respectively) and contained a nearly full complement of metal (Fe/β\(_i\) = 3.8 ± 0.2; Mn/β\(_i\) = 3.8 ± 1.6) and Y- (Fe-β\(_i\) = 1.2 Y/β\(_i\); Mn-β\(_i\) = 0.7 Y/β\(_i\)).

\[ K_m \text{ for } \alpha/\beta \text{ Interaction.} \] Recent Western blot analysis of wild type B. subtilis JH624 using antibodies to NrdE and NrdF revealed that the two proteins were present in the crude cell extracts in a 1:1 subunit ratio at ~1 μM.9 Thus, CDP reductase activity using a 1:1 subunit ratio was examined with subunit concentrations that ranged from 0.01 μM to 1 μM in the presence of ATP (3 mM) and TrxA/TrxB/NADPH. The results are shown in Figure 4. Fitting the data to eq 1 gave \( V_{\text{max}} = 124 ± 6 \text{ U/mg and } K_m = 0.06 ± 0.01 \mu M \text{ for Fe-β}_i \text{ and } V_{\text{max}} = 1081 ± 36 \text{ U/mg and } K_m = 0.025 ± 0.003 \mu M \text{ for Mn-β}_i \). The apparent affinity between the subunits is ~10χ greater than for the class Ia RNRs and the B. anthracis class Ib RNR.13,23,24

**Quaternary Structure by SEC.** SEC was used initially to estimate the molecular weights of the subunits and quaternary structure(s) of holo-RNR. When run at physiological concentrations (~1 μM), NrdF (Fe-, or Mn-loaded) eluted predominantly as a single peak (Figure 5A) with a molecular weight of 80.1 kDa (Table 2), consistent with a dimer. A small amount of monomer was also observed (49.6 kDa, Table 2). NrdE also eluted as a single peak (Figure 5A) with a molecular weight of 82.7 kDa (Table 2), consistent with a monomer. The results with holo-RNR are shown in Figure 5B. The chromatogram reveals a series of broad peaks with retention times ranging from 37–60 min, indicating the presence of interconverting species. Regardless of the metal loading, the highest percentage of protein eluted at \( V_e = 47 \text{ min, corresponding to a molecular weight of 204.6 kDa (Table 2), suggesting, in conjunction with the other hydrodynamic properties, the presence of an } \alpha/\beta_2 \text{-like complex with a structure similar to the elongated } S. \text{ typhimurium class Ib holo-RNR (Table 2).}^{31} \text{ However, these results clearly indicate that the quaternary structure(s) of the } B. \text{ subtilis class Ib RNR are not stable in the absence of substrate or effector nucleotides, resulting in an ensemble of different interconverting species, despite the low experimentally measured } K_m \text{ (Figure 4).}

**Quaternary Structure by SV-AUC.** SV experiments with each subunit and with a 1:1 mixture of subunits were carried out at different concentrations (Supporting Information, SI-Table 3) to gain further insight into their quaternary states. To aid in the analysis of our experimental data, HYDROPRO\(^33\) was used to predict hydrodynamic properties for NrdF and NrdE.
from the NrdF crystal structure (PDB 4DRO)\(^{44}\) and a threading model of NrdE using the \textit{S. typhimurium} NrdE structure (PDB 1PEM)\(^{45}\). Predictions were also made for docking models of the \textit{B. subtilis} \textit{αββ} complex prepared by \textit{in silico} alignment of the subunit models with the \textit{E. coli} class Ia RNR globular docking model and the elongated X-ray structure of the \textit{S. typhimurium} class Ib holoprotein\(^{51}\). The results of the HYDROPRO calculations are summarized in Supporting Information, SI-Table 4.

The sedimentation behavior of Mn\(^{111}YG\cdot\text{NrdF} (1−11 \text{ μM}), \text{NrdE} (1−30 \text{ μM}), and \text{NrdE:Fe}\(^{II}YG\cdot\text{NrdF} (25 \text{ nM to } 7 \text{ μM}) were determined and analyzed using the \textit{c(s)} model in Sedfit and, due to the detection by the \textit{c(s)} fits of interactions occurring on the time scale of sedimentation, with Sedphat. The details of these analyses are presented in the Supporting Information and in SI-Figures 5–7. The results for 1 \text{ μM Mn}\(^{111}YG\cdot\text{NrdF} are shown in Figure 5C and reveal two peaks representing stable, noninteracting entities at all concentrations examined (Supporting Information, SI-Figure 5). Globally fitting the data sets in Sedphat yielded \(s_{20,w} = 3.64 \text{ and } 4.72 \text{ S for each entity, along with the corresponding } M_n\)'s listed in Table 2. These results, in comparison with HYDROPRO predictions (Supporting Information, SI-Table 4) and SEC results (Table 2), suggest that the predominant species is a dimer and the minor species is a monomer. These results are consistent with the general reports that all class Ia small subunits are dimers.

The results of similar experiments with NrdE are shown in Figure 5C and Supporting Information, SI-Figure 6. At 1 \text{ μM NrdE}, a single peak with an \(s_{20,w} = 5.56 \text{ S is observed. However, analysis of increasing protein concentrations to } 9 \text{ μM reveal a second peak at } s_{20,w} = 7.40 \text{ S (Supporting Information, SI-Figure 5A) and at still higher concentrations (30 μM), larger oligomeric states (Supporting Information, SI-Figure 5B). Thus, NrdE is in a regime where the protein is not stable and aggregates on the time scale of our experiments. The NrdE data analyzed in Sedphat, when compared to the HYDROPRO predictions (Supporting Information, SI-Table 4) and our SEC data (Table 2), suggest that the peak at low concentrations is the NrdE monomer. As the protein concentration increases NrdE appears to form a dimer that is unstable and continues to aggregate (Supporting Information, SI-Figure 6). We note that \(α\) in prokaryotic and eukaryotic class Ia RNRs, in general exhibits low solubility and that aggregation has been reported by SAXS and EM analyses.\(^{46}\) However, ours is the first systematic report of the complexity of \(α\) aggregation.

The analysis of a 1:1 mixture NrdE:Fe\(^{II}YG\cdot\text{NrdF using } c(s)\text{ is shown in Figure 5D (also see Supporting Information, SI-Figure 7). Even at 1 μM, dynamic behavior of the subunits is indicated by the reaction boundary that has apparent peaks at 4.38 and 9.66 S. A plot of the best fit \(f/f_o\) from the \(c(s)\) models versus loading concentration (Figure 5E) reveals a sharp decrease as the protein concentration increases. As described in more detail in the Supporting Information, this observation indicates the presence of interconverting species. The data were imported into Sedphat, and the peaks were treated as discrete species with individual \(f/f_o\).\(^{40}\) The calculated hydrodynamic properties of the smaller species (\(s_{20,w} = 3.64 \text{ S}) are consistent with a NrdF monomer. For the larger species (\(s_{20,w} = 9.59 \text{ S})\), the analysis suggests the presence of oligomeric structures that are similar to, but smaller than, that predicted for an \(αβ\) complex (Supporting Information, SI-Table 4). As noted however, this is clearly not a discrete species as this peak shifts to larger \(s_{20,w}\) and larger complex(es) of unknown composition become evident as the protein concentration increases (Figure 5F, Supporting Information, SI-Figure 7). Thus, we currently do not have a model for the behavior of the 1:1 NrdE:NrdF complex. Further analysis requires the presence of different substrate and effector pairs after their binding stoichiometries are established and requires development of a method to specifically reduce the Y\(^{-}\) in NrdF to prevent chemistry during the analysis.\(^{34}\)

### DISCUSSION

In 2010, it was shown that class Ib RNRs can assemble a Mn\(^{111}YG\cdot\text{cofactor with the aid of the unusual flavodoxin Nrdl and O}_2\text{ in vitro}\(^{38}\) and that enzymes isolated from three endogenous sources have a similar cluster.\(^{6,7,9}\) More recently, \textit{in vitro} assembly of a Mn\(^{111}YG\cdot\text{cofactor using species specific NRdls have been carried out in } B.\text{ subtilis,}\(^{9,27}\) Streptococcus sanguinis,\(^{53}\) \textit{B. anthracis}, and \textit{B. cereus}\(^{54}\) with similar results. The interesting observation is that a Fe\(^{II}YG\cdot\text{cofactor can self-assemble in these enzymes in vitro and maintain catalytic activity, although demonstrably lower than the Mn-loaded cofactor (Supporting Information, SI-Table 1),}\(^{3–5,46,51}\) raises important biological questions as to the nature of the active cofactor \textit{in vivo} and whether it changes with the growth conditions of the organism. Given that many pathogenic organisms\(^ {54}\) require a class Ib RNR for aerobic growth and that humans use a Fe\(^{II}YG\cdot\text{class Ia RNR, the distinctions in the mechanisms of cofactor biosynthesis, allosteric regulation, and quaternary structure offer new opportunities for therapeutic intervention. Thus, understanding
the basic properties of the Ib RNRs, as well as their similarities and differences to one another and to class Ia RNRs, is an important first step.

Recent studies on the class Ib RNRs (with the Mn\(^{III}\)·Y-NrdF) from B. anthracis and B. cereus have identified and characterized the endogenous reductants required to make deoxynucleotides at optimum rates.\(^{24,25}\) Despite this important contribution, however, these and other class Ib RNRs (Supporting Information, SI-Table 1) still have low catalytic activity. The B. anthracis Mn\(^{III}\)·Y-NrdF assembled with NrdI gave 0.3–0.4 Y/\(\beta_2\) but the reported activity was only 44–70 U/mg.\(^{24,25}\) Similar activities were observed using DTT (40–63 U/mg) alone or with either TR or NrdH.\(^{24,25}\) The B. anthracis F\(^{III}\)·Y-NrdF with 0.6 Y/\(\beta_2\) was reported to have activity of only 8 U/mg.\(^{25}\) This value is very low given the high Y-content, which in the case of the Ia RNRs, is correlated with activity.\(^{35}\) Our initial report on the activity of endogenous B. subtilis RNR using DTT as reductant was also low, 160 U/mg. To study the class Ib RNRs, we thus felt that we needed to better understand the basis for the low catalytic activity, which we believed to lie with cluster assembly in NrdF and the reductant used to support NDP reduction. Our studies show that apo-/mismetalated forms of NrdF can be separated from holo-protein, yielding Mn\(^{III}\)·Y-NrdF with 1 Y/\(\beta_2\) and 4 Mn/\(\beta_2\). Contrary to our expectations, the purity of Mn\(^{III}\)·Y-NrdF was only a minor factor in activity enhancement (Table 2). Rather, the identity of the reductant used to support turnover was the major limiting factor in obtaining highly active RNR from B. subtilis.\(^{37}\) The optimum endogenous reducing system was TR/TRR/NADPH, which supported catalytic activity of ~1450 U/mg. However, the major contributor to high enzymatic activity may vary from species to species, given that with B. anthracis, TrxA and DTT yielded similar activities using poorly loaded Mn\(^{III}\)·NrdF.\(^{25}\) The basis for the low activity in the B. anthracis system is likely associated with mismetalated NrdFs which interfere with formation of an active RNR complex.

The availability of highly active B. subtilis NrdF and NrdE have allowed us to assemble an active RNR with a 1:1 ratio of subunits and to analyze the resulting activity in physiological concentration ranges (Figure 4). The \(K_a\) of 0.025 \(\mu M\) contrasts with values of ~0.2 \(\mu M\) measured for the Ia RNR subunits, in which each subunit is assayed independently in the presence of a 5–10-fold excess of the second subunit to generate the "active" complex.\(^{13,14}\) All the assays on the B. anthracis RNR were carried out using the class Ia method.\(^{24,25}\) These conditions could alter quaternary structures (\(\alpha/\alpha\) and \(\alpha/\beta\) structures) and result in inhibition under these assay conditions (see discussion below). With bacterial systems, we now believe that all assays of enzymatic activity should be carried out with a 1:1 ratio of the two subunits, accompanied by a study to establish the optimum concentration range for maximum active complex formation with each RNR.

Two issues warrant further discussion of our results with B. subtilis in comparison those with B. anthracis (and B. cereus) Ib RNRs: the nature of the endogenous reductant(s) and the nature and dynamics of their quaternary structure (s). In the studies of Gustafsson et al.,\(^{17,22}\) interrogation of the B. anthracis genome identified three thioredoxins, two potential thioredoxin reductases, and three potential glutaredoxin-like proteins all of which were cloned, expressed, and screened for their ability to support RNR activity.\(^{25}\) The turnover numbers for the B. anthracis RNR were highest with Trx1/TR1 and 7-fold greater than with NrdH/TR1 (Supporting Information, SI-Table 1). Furthermore, under their growth conditions, Western analysis revealed that TrxA was the predominant RNR reductant in the cell.\(^{25}\)

We used the B. anthracis genes\(^{24,25}\) as queries of the B. subtilis genome and identified six thioredoxin-like proteins (TrxA, Ydpb, YtpP, YdfQ, YusE, and Yus1), one thioredoxin reductase (TrxB), and two glutaredoxin-like proteins (YosR and BdbA). TrxA and TrxB are homologues of B. anthracis TrxI (75% identity) and TR1 (87% identity), suggesting they likely function as the reducing system for the B. subtilis RNR. In addition, previous gene knock-out experiments of all of these proteins and the growth of the resulting deletion mutants in rich or minimal medium revealed that only TrxA and TrxB are essential.\(^{10,56}\) Using these purified proteins, we established that endogenous reductants effect a ~10-fold increase on RNR activity relative to DTT (Table 1) in contrast with the B. anthracis RNR.\(^{25}\) We also note that we have observed a 20-fold difference between endogenous reductant and DTT with the S. sanguinis class Ib RNR.\(^{33}\)

It was pointed out previously that B. subtilis has a second class Ib RNR, located in the bnrdeFI operon, which could potentially be physiologically important.\(^{34}\) The gene yosR, recently reannotated as nrdH, is adjacent to this operon,\(^{25,34}\) prompting us to examine this protein in our assays as well. As was observed with the B. anthracis RNR, YosR(NrdH) exhibits activity similar to TrxA in ribonucleotide reduction (Table 1). In B anthracis, as noted above, Western analysis suggested that TrxA was the endogenous reductant. Previous studies in B. subtilis have established that the bnrdeFI operon is dispensable for its survival under numerous growth conditions,\(^{11,12,57}\) supported by transcriptional analysis under rich and minimal growth conditions in which both yosR and bnrdeFI were expressed at very low levels.\(^{58}\) Thus, TrxA is most likely the physiological reductant in B. subtilis as well.

The most interesting observation from our studies is associated with our analysis of quaternary structure. We expected tight subunit association in the absence of nucleotides (dNTPs), based on our isolation of endogenous B. subtilis RNR\(^{7}\) and the concentration dependence of activity (Figure 4), in contrast to the class Ia RNRs (\(K_a\) of 0.4 \(\mu M\)).\(^{14,59}\) Our studies using SEC and SV-AUC, however, revealed that this is not the case (Figure 5). Under physiological concentrations (1 \(\mu M\) with no nucleotides, both methods revealed mixtures of subunits and complexes of subunits. GEMMA analysis of the B. anthracis class Ib RNR in the absence of nucleotides also revealed mixtures of subunits and an \(\alpha_2\beta_2\) complex.\(^{24}\) In neither our case, nor in the case of these earlier studies, however has a study of affinities and the rates for subunit association and dissociation been carried out.

Finally, the observation of potent inhibition of RNR activity by dATP given the absence of an ATP cone domain deserves comment. The results are distinct from all class Ib RNRs reported to date, which show no inhibition even at concentrations of 1 mM dATP.\(^{5,49,50,53,60}\) Furthermore, with dATP and the B. anthracis RNR, the GEMMA analysis revealed individual subunits, \(\alpha_2\beta_2\) and \(\alpha_2\beta_2\), but no large quaternary states were reported.\(^{24}\) Thus, the cause for the potent inhibition with the B. subtilis RNR requires further study.

Our studies\(^{16,17,22}\) suggest that the paradigm of RNR as \(\alpha_2\beta_2\) in the literature needs to be re-examined. The difficulties associated with obtaining structures of prokaryotic RNRs with both subunits present are likely associated with the dynamics of
their interactions. Undoubtedly these dynamics will be affected by the presence of ATP, dNTPs, and Mg2+, and studies of these effects under physiologically relevant conditions are currently in progress. In order to carry out these types of studies, highly active protein with fully loaded metallo-cofactor is essential, and our studies reported herein present the foundation to investigate quaternary structure and its connection to the enzymatic activity and allosteric regulation of RNRs.

### ASSOCIATED CONTENT

**Supporting Information**

Experimental details of NrdE purification, SDS-PAGE densitometry, and hydrodynamic property predictions; detailed results of SV-AUC experiments; TrxA, TrxB, and YosR cloning primers and purity analyses; spectroscopic characterization of apo-/mismetalated and holo-MeIII

### ACKNOWLEDGMENTS

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### ABBREVIATIONS

dATP, deoxyadenosine S′-triphosphate; ATP, adenosine S′-triphosphate; BCA, bicinchoninic acid; BSA, bovine serum albumin; CDP, cytidine S′-diphosphate; CV, column volume; dNDPs, deoxyribonucleoside S′-diphosphates; dNTPs, deoxyribonucleoside S′-triphosphates; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetraacetic acid; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; HEPES, N-2-hydroxyethylpipеразине-Ν-2′-еthenesulfonic acid; IPTG, isopropyl-β-D-thiogalactoside; NADPH, nicotinamide adenine dinucleotide phosphate; NaP, sodium phosphate; NDps, ribonucleoside-S′-diphosphates; Ni-NTA, nickel nitritolriatic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SV-AUC, sedimentation velocity analytical ultracentrifugation; TCEP, tris(2-carboxyethyl)phosphine; TR, thioredoxin; Tris, Tris-

(hydroxyethyl)amino methane; TRR, thioredoxin reductase; Y, tyrosyl radical

### ADDITIONAL NOTE

“The reported activity of the C. ammoniagenes class Ib RNR is inconsistent with activities of all known RNRs and is likely incorrect.

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**NOTE ADDED AFTER ASAP PUBLICATION**

This article was published ASAP on January 21, 2014, with minor errors in Table 2. The corrected version was published ASAP on January 22, 2014.