Supporting Information for:

*Bacillus subtilis* Class Ib Ribonucleotide Reductase:

High Activity and Dynamic Subunit Interactions

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References
Supplementary Materials and Methods

Purification of NrdE. The following modifications were introduced into our previously published procedure (1). NaCl was added to the lysis buffer to a final concentration of 300 mM and 150 mM in the Ni-NTA elution buffer. NrdE isolated from the affinity column was further purified using anion exchange chromatography. Protein was loaded onto a Q-Sepharose column (5 x 2.5 cm) equilibrated 50 mM Tris, pH 7.6 (adjusted at 4 °C), 5% (w/v) glycerol, 10 mM DTT and 100 mM NaCl. The column was washed with 2 CVs of equilibration buffer before elution with a 250 mL linear gradient from 100 to 500 mM NaCl in Tris buffer. NrdE eluted between 300-350 mM NaCl. Fractions containing NrdE were identified by SDS-PAGE, pooled, desalted, and exchanged into storage buffer (50 mM sodium phosphate, 150 mM NaCl, 5% (w/v) glycerol, 10 mM DTT, pH 7.6) using an Amicon Ultra-15 YM-50 centrifugal filter (Millipore, 50000 MWCO). Purity was assessed on 7% SDS-PAGE gels and protein concentrations were estimated using ε_{280 nm} = 79100 M⁻¹ cm⁻¹ calculated from the amino acid sequence of the His-tagged protein using the ProtParam tool of the ExPASy Bioinformatics Resource server (2) (http://web.expasy.org/protparam/).

Prediction of the hydrodynamic properties of the B. subtilis class Ib RNR. The web server PHYRE was used to generate a tertiary structure prediction of BsNrdE from the experimentally determined S. typhimurium NrdE structure (PDB 1PEM) (3, 4). Pairwise BLAST alignment of the BsNrdE (accession # NP_389620) and StNrdE (accession # AAL21692) sequences revealed 45% identity and 65% positive substitution between the two proteins, and produced 10 gaps (1% of total sequence). Two models of the B. subtilis α₂β₂ complex were prepared and analyzed using UCSF Chimera (5). In one model, two BsNrdE monomers and the Mn^{II}_{2}-BsNrdF dimer (PDB 4DRO) (6) were aligned with the docking model of the E. coli class Ia RNR (RMSD = 1.1 Å over 400 atom pairs) (7). In the second model they were aligned with the structure of the class Ib RNR
holo-complex from *S. typhimurium* (PDB 2BQ1, RMSD = 1.2 Å over 474 atom pairs). The sedimentation coefficient at standard state (s_{20,w}) of α, β, α_2, β_2, and the α_2β_2 complex were calculated from an atomic-level shell model using the program HYDROPRO under default settings using ρ = 0.998 g mL\(^{-1}\), and η = 0.01 poise, and the molecular weights (M_w) and partial specific volumes (\(\bar{\upsilon}\)) listed in SI-Table 4, all calculated and temperature corrected in Sednterp. The theoretical frictional coefficients (f) were calculated using eq 2, where \(N_A\) is Avogadro’s number. The Stokes radii (R_s) were calculated from f using eq 3.

\[
f = \frac{M_w(1-\bar{\upsilon}\rho)}{N_A s_{20,w}} \tag{2}
\]

\[
f = 6\pi\eta R_s \tag{3}
\]

The maximum expected sedimentation coefficients (s_{max}) for each component at 20 °C in water were calculated using eq 2 rearranged for \(s_{20,w}\), with the minimum frictional coefficient (f_o) determined by substituting R_s with \(\left(\frac{3M_w}{4\pi N_A}\right)^{\frac{1}{3}}\) in eq 3.

**SDS-PAGE densitometry.** Samples of 1:1 mixtures of α and β (~4 μM, 10 – 20 μL) were mixed with an equiv. volume of 2X Laemmli buffer supplemented with 3% (v/v) BME and incubated at ~100 °C for 5 min. The samples were centrifuged (14100 x g, 1 min, RT) and 10 – 20 μL were loaded per well on 18 well, 10% Tris-HCl Criterion denaturing gels (BioRad). NrdE and NrdF standards (10, 20, 30, 40, and 50 pmol in 25 μL) were loaded onto the same gel. Gels were run at 4 °C and 180 V, stained with Coomassie Brilliant Blue R-250, and imaged on a Gel Doc™ 2000 Imager (BioRad). Band intensities were quantified using Quality One software (BioRad), and the relative amounts of NrdE and NrdF in samples of complex were determined using the corresponding linear fits generated from the respective protein standard curves.
Supplementary Results

*SV-AUC results for holo-Mn\textsuperscript{III}\textsubscript{2}-Y• NrdF.* Over the entire concentration range tested (0.8 – 10.9 µM), the \(s_{20,w}\) distributions of Mn\textsuperscript{III}\textsubscript{2}-Y• NrdF displayed two peaks with average weighted sedimentation coefficients (\(s_{w(20,w)}\)) of 3.29 S and 4.77 S ([SI-Figure 5A](#)). The signal contributions of the two peaks remained constant over the entire concentration range, indicating two non-interacting species were present in solution. Globally fitting all data sets in Sedphat using the “hybrid local continuous/global discrete species model” yielded \(s_{20,w}\) values of 3.64 S (~20% of the total signal) and 4.74 S (~80% of the total signal), and \(M_w\) values of 35.4 and 94.2 kDa ([Table 2](#)). These values, along with the frictional ratio \((f/f_o)\) and \(R_s\) determined in Sednterp with the global \(s_{20,w}\) values, clearly indicate that the larger species corresponds to NrdF dimer and the smaller species to NrdF monomer (likely apo- or mismetallated) when compared with the SEC results ([Table 2](#)) and the predictions made by HYDROPRO ([SI-Table 4](#)). Large deviations between the experimentally measured and predicted hydrodynamic properties can be expected since the crystal structure of NrdF used to make the predictions is incomplete (missing the N-terminal His-tag and the C-terminal tails of each \(\beta\) unit). Additionally, apo-NrdF may not have the same structure as holo-NrdF, and since a structure of the apo-protein is not available, deviations in the predicted and measured parameters for this protein can be expected. One final note is that the peak corresponding to Mn\textsuperscript{III}\textsubscript{2}-Y• NrdF dimer appears to decrease in \(s_{20,w}\) as the protein concentration increases, which is clearly evidenced by the decreasing slope in the weight averaged \(s_{20,w}\) isotherm ([SI-Figure 5B](#)) and suggests the protein behaves non-ideally in solution. The weight averaged \(f/f_o\) ([SI-Figure 5C](#)) from the c(s) models remains fairly constant at about 1.4, further confirming the absence of self-association reactions and indicating the protein shape, while slightly
elongated relative to the predictions from HYDROPRO (Table 2), is mostly globular over the entire concentration range.

**SV-AUC results for NrdE.** Using $A_{280}$ as the detection wavelength, the detectable concentration range of NrdE was 1 – 12 µM (SI-Table 3). At physiological concentrations (~ 1 µM), NrdE, in the absence of nucleotides, sedimented as a single peak ($s_{w,20} = 5.56$, SI-Figure 6A) with hydrodynamic properties consistent with NrdE monomer (Table 2). As the concentration of NrdE was increased, the peak broadened and eventually evolved into a reaction boundary at ~9 µM NrdE consisting of two peaks with $s_{20,w} = 5.64$ S and 7.40 S (SI-Figure 6A). These values and the associated hydrodynamic properties and $M_w$ are consistent with NrdE monomer and dimer (Table 2). To probe the self-association of NrdE at higher concentrations, $A_{250}$ detection was used where the estimation of protein concentration was obtained using the $A_{280}$:$A_{250}$ ratio (2.54 ± 0.20). The range detectable using this wavelength was 3 – 40 µM. In this concentration regime, the peak corresponding to $\alpha_2$ shifted to ~9.7 S and broadened into a second reaction boundary at the highest concentrations tested (27 – 32 µM NrdE) with the larger sedimenting species appearing at ~12 S (SI-Figure 6B). The isotherm of the weight averaged $s_{20,w}$ plotted against the entire concentration range analyzed (SI-Figure 6C) never reaches an upper asymptote, appearing to increase exponentially from a lower asymptote of ~5.8 S. The $\alpha$ subunits of the class I RNRs have long been known to have a propensity to form aggregates, and the results reported here, while not surprising, provide the first systematic study of this. The weight averaged $f/f_o$ decreased from ~1.25 at 1 µM NrdE to ~0.90 at 32 µM (SI-Figure 6D). Given that the physically possible minimum $f/f_o$ is 1.0 for a perfect sphere, these unrealistic $f/f_o$ indicate extra boundary broadening during sedimentation is not attributable to diffusion alone. These values instead indicate NrdE self-association reactions are fast relative to the timescale of sedimentation.
**SV-AUC analysis of 1:1 NrdE:Fe\textsuperscript{III}-Y• NrdF mixtures.** As mentioned in the main text, a reaction boundary with apparent peaks at 4.38 S and 9.66 S was observed at 1.2 µM (Figure 5D). These values are not necessarily the true $s_{20,w}$ of these entities as each protein exhibits a different $f/f_o$, but the c(s) model only employs a single, weight averaged $f/f_o$ to fit the data. Protein interactions and their kinetics relative to the timescale of sedimentation influence the fitting of this ratio, typically leading to under or over estimation of the $s_{20,w}$ for the actual species corresponding to the peaks displayed in the distributions. A plot of the best fit $f/f_o$ from the c(s) models versus loading concentration (Figure 5E) for the entire collection of SV experiments with 1:1 NrdE:NrdF mixtures shows a sharp decrease in value as the protein concentration increases. As described above for NrdE, this likely indicates extra boundary broadening resulting from protein interactions that are rapid relative to the timescale of sedimentation \((12)\). To try to get a better idea of the molecular weights and hydrodynamic properties of the entities, the data for the 1.2 µM sample was fit for $s_{20,w}$ and $M_w$ (Table 2) in Sedphat by treating the peaks as discrete species with individual $f/f_o$ that are surrounded by segments of continuous distribution that can be fitted for their own ratios \((12)\). In order to determine the more precise $f/f_o$ (and $R_s$) for each species, the best fit $s_{20,w}$ were entered into the program Sednterp, using the $M_ws$ expected from amino acid sequence, as those determined by Sedphat by solely fitting SV-AUC data in Sedphat are typically ± 10% of the actual value. The calculated hydrodynamic properties of the smaller species (Table 2), in comparison with the SEC data and HYDROPRO predictions (SI-Table 4), strongly suggest that it corresponds to NrdF monomer. The results for the larger species ($s_{20,w} = 9.59$ S) are similar to those of the large species observed in the SEC data (Table 2), but are smaller than predicted for an $\alpha_2\beta_2$ complex (SI-Table 4). The formation of these larger complexes prevent rigorous treatment of the data to determine the kinetics and affinities of the binding partners, which can be estimated.
by plotting the weight averaged $s_{20,w}$ versus loading concentration to generate a binding isotherm. As shown in Figure 5F, the weight averaged $s_{20,w}$ isotherm is complicated and we currently do not have a model that would satisfactorily explain the data.
**SI-Table 1.** Activities of metal loaded NrdFs from various sources.

| Source            | Metal loading | Y•/β₂  | Reductant           | Equiv. of α | SA (U mg⁻¹) | Ref. |
|-------------------|---------------|--------|---------------------|-------------|-------------|------|
| *B. anthracis*    |               |        |                     |             |             |      |
| Reconstituted Fe  | 0.57          | TR/TRR/NADPH | ~0.4              | 4 – 8       | 7.2 – 9.6   | (13) |
|                   |               | DTT    |                     | 4 – 8       | 8.4 – 8.9   | (13) |
| Reconstituted Mn  | 0.3 – 0.4     | TR/TRR/NADPH | ~0.4              | 4 – 8       | 56 – 70     | (13) |
|                   |               | DTT    |                     | 4 – 8       | 27 – 40     | (13) |
| *B. subtilis*     |               |        |                     |             |             |      |
| As isolated Mn    | 0.4 – 0.5     | DTT    |                     | 10          | 160         | (1)  |
| Reconstituted Fe  | 1 – 1.2       | TR/TRR/NADPH | 1              | 156         | This study  |      |
|                   |               | DTT    |                     | 1           | 52          | This study |
| Reconstituted Mn  | 1.1           | TR/TRR/NADPH | 1              | 1475        | This study  |      |
|                   |               | DTT    |                     | 1           | 146         | This study |
| *C. ammoniagenes* |               |        |                     |             |             |      |
| As isolated Fe    | 0.05          | DTT    |                     | 1 – 4       | 36          | (15) |
| As isolated Mn    | 0.36          | DTT    |                     | 2           | 69000^d     | (16) |
| Reconstituted Fe  | 0.2           | DTT    |                     | 1 – 4       | 48          | (15) |
| Reconstituted Mn  | 0.03          | DTT    |                     | 1 – 4       | 9           | (15) |
| *E. coli*         |               |        |                     |             |             |      |
| As isolated Mn    | 0.2           | DTT    |                     | 5           | 720         | (17) |
| Reconstituted Fe  | 0.7           | DTT    |                     | 5           | 300         | (18) |
| Reconstituted Mn  | 0.25          | DTT    |                     | 5           | 600         | (18) |
| *Lactococcus lactis* |       |        |                     |             |             |      |
| As isolated N/R   | N/R^c         | DTT    |                     | N/R^c       | 198         | (19) |

^a^ Data for reconstituted enzyme

^b^ Data for as isolated enzyme

^c^ Data not reported

^d^ Data reported in units of 10⁴
**SI-Table 1. (continued)**

| Organism                      | As isolated<sup>b</sup> | Fe    | DTT  | TR/TRR/NADPH |
|-------------------------------|-------------------------|-------|------|---------------|
| *M. tuberculosis*             |                         | 0.3 – 0.4 | 1 – 32 | 120            |
| *Staphylococcus aureus*       |                         | N/R<sup>c</sup> | N/R<sup>c</sup> | (21)           |
| Reconstituted                 | N/R<sup>c</sup>        | N/R<sup>c</sup> | 2     | 48            |
|                               |                         | 2      | 61   | (21)           |
| *Streptococcus pyogenes*      |                         | 1      | DTT  | N/R<sup>c</sup> |
| As isolated<sup>b</sup>       |                         | 0.9    | 0.05 – 3 | 169            |
| *S. typhimurium*              |                         | 0.4    | DTT  | Reconstituted |
| As isolated<sup>b</sup>       |                         | 0.4    | 0.5    | 325            |
|                               | Reconstituted           | Fe     | DTT  | 1 – 4 |

<sup>a</sup> Isolated from the endogenous species.  <sup>b</sup> Isolated from *E. coli* overexpression strains.  <sup>c</sup> N/R = not reported.  <sup>d</sup> This reported activity is dubious as it is substantially higher than any other class I RNR activity reported. The strain of *C. ammoniagenes* genetically manipulated to overexpress NrdF is no longer available, thus this activity cannot be confirmed.

**SI-Table 2.** Cloning primers for *B. subtilis* *trxA*, *trxB*, and *yosR*.

| Gene | Primer direction | Sequence |
|------|------------------|----------|
| *trxA* | Sense | 5'-AGT CAG CAT ATG ATG GCT ATC GTA AAA GCA ACT GAT |
|       | Antisense | 5'-AGT CAG CTC GAG TCA AAG ATG TTT GTT TAC AAG CTC |
| *yosR* | Sense | 5'-AGT CAG CAT ATG ATG AGA TTA ATT AAA TTA GAG CAG |
|       | Antisense | 5'-AGT CAG CTC GAG TCA TCG TAA TTC CTT TAG TAA CTC |
| *trxB* | Sense | 5'-AGT CAG CAT ATG GTG TCA GAA GAA AAA ATT TAT GAC |
|       | Antisense | 5'-AGT CAG CTC GAG TCA TTT TAA GGT TTT CAG CGT TTC |

NdeI and XhoI sites are underlined. Primers were designed such that the native start codons (ATG for *trxA* and *yosR*, GTG for *trxB*) were retained in the clones. The His-tagged TrxA and YosR produced from these constructs contain an extra methionine residue between the 20 residue His-tag-linker and the original start codon of the respective protein. In the case of TrxB, the extra amino acid is valine. The native ochre stop codon was changed to an opal stop codon.
**SI-Table 3.** Concentration ranges used for SV-AUC analysis of *B. subtilis* class Ib RNR.

| Sample           | Concentrations (μM)          |
|------------------|------------------------------|
|                  | A280: 1.0, 2.4, 4.3, 6.7, 8.9, 11.4 |
|                  | A250: 12.3, 14.7, 16.6, 18.9, 26.7, 32.3 |
| Mn-β2            | A280: 0.8, 1.6, 3.4, 5.2, 8.1, 10.9 |
| α + Fe-β2<sup>a</sup> | A230: 0.026, 0.042, 0.064, 0.23, 0.56, 0.67 |
|                  | A280: 0.48, 1.2, 2.0, 2.7, 3.6 |
|                  | A250: 4.8, 5.3, 6.2, 6.8 |

<sup>a</sup> Sample was a 1:1 mixture of α:β.

**SI-Table 4.** HYDROPRO<sup>(9)</sup> predictions of hydrodynamic properties of components of the *B. subtilis* class Ib RNR.

| Subunit/complex | $\bar{v}$ (cm$^3$ g$^{-1}$)<sup>a</sup> | M.W. (kDa)<sup>b</sup> | Rs (Å) | $s_{20, w}$ (S)<sup>c</sup> | $f \times 10^{-7}$ (gs$^{-1}$)<sup>f</sup> | $f_{o} \times 10^{-7}$ (gs$^{-1}$)<sup>d</sup> | $f/f_o$ |
|-----------------|------------------------------------------|------------------------|--------|---------------------------|-----------------------------------------------|-----------------------------------------------|--------|
| α               | 0.7315                                   | 83                     | 36.4   | 5.4                       | 0.692                                         | 6.8                                           | 0.545  | 1.27  |
| β               | 0.7352                                   | 41                     | 27.2   | 3.5                       | 0.518                                         | 4.5                                           | 0.431  | 1.20  |
| α<sub>2</sub>   | 0.7315                                   | 166                    | 48.0   | 8.2                       | 0.903                                         | 10.8                                          | 0.687  | 1.31  |
| β<sub>2</sub>   | 0.7352                                   | 81                     | 34.6   | 5.5                       | 0.652                                         | 6.6                                           | 0.542  | 1.20  |
| α<sub>2</sub>β<sub>2</sub> | 0.7327                                  | 247                    | 51.2<sup>e</sup> (54.0)<sup>f</sup> | 11.4<sup>e</sup> (10.8)<sup>f</sup> | 0.967<sup>e</sup> (1.021)<sup>f</sup> | 14.1                                           | 0.785  | 1.23<sup>e</sup> (1.30)<sup>f</sup> |

<sup>a</sup> Calculated with 20 °C temperature correction in Sednterp<sup>(10)</sup>.

<sup>b</sup> Based on amino acid sequence.

<sup>c</sup> Predicted from crystal structures in HYDROPRO using $\rho = 0.998$ g cm$^{-3}$ and $\eta = 0.01$ poise.

<sup>d</sup> Maximum sedimentation and frictional coefficients expected for a perfect sphere of the same molecular weight.

<sup>e</sup> Based on alignment of *B. subtilis* class Ib RNR with the *E. coli* class Ia docking model<sup>(7)</sup>.

<sup>f</sup> Based on alignment of *B. subtilis* class Ib RNR with the *S. typhimurium* holo-RNR complex<sup>(8)</sup>.
**SI-Figure 1.** Purification of His-tagged versions of TrxA, YosR, and TrxB. (A) TrxA isolation. Fractions were resolved on a 17% SDS-PAGE gel. Lane 1: insoluble fraction, lane 2: cell free extract, lane 3: streptomycin pellet, lane 4: streptomycin supernatant, lane 5: Ni-NTA loading flow through, lane 6: Ni-affinity column eluate, lane 7: 6 μg Sephadex G-75 purified TrxA. (B) YosR refinement using a Poros HQ/20 anion exchange column. Samples were resolved on a 15% SDS-PAGE gel. Lane 1: Ni-affinity column eluate, lane 2: Poros HQ/20 eluate. (C) TrxB refinement using a Poros HQ/20 anion exchange column. Samples were resolved on a 15% SDS-PAGE gel. Lane 1: Ni-affinity column eluate, lane 2: Poros HQ/20 eluate.

**SI-Figure 2.** MonoQ anion exchange chromatography of a 1:1 ratio holo-Fe$^{III}$-Y-NrdF and NrdE (5 μM final subunit concentrations).
SI-Figure 3. UV-visible and EPR spectra of MonoQ separated holo- and apo-NrdF. Key: **Black traces** = Peak 2 fractions; **Red traces** = Peak 1 fractions.

(A) UV-vis spectra of fractions collected from a Mn$^{III}_2$-Y• reconstituted NrdF sample. *Inset:* Blow up of the region between 300 – 800 nm. $\lambda_{Y}\cdot = 390$ nm, 410 nm. $\lambda_{\text{dimagamic cluster}} = 450 – 700$ nm.

(B) EPR spectra (77 K) of fractions collected from a Mn$^{III}_2$-Y• reconstituted NrdF sample.

(C) UV-vis spectra of fractions collected from a Fe$^{III}_2$-Y• reconstituted NrdF sample. *Inset:* Blow up of the region between 300 – 500 nm. $\lambda_{Y}\cdot = 410$ nm. $\lambda_{\text{diferric cluster}} = 320$ nm, 360 nm.

(D) EPR spectra (77 K) of fractions collected from a Fe$^{III}_2$-Y• reconstituted NrdF sample.
SI-Figure 4. SDS-PAGE densitometric analysis of fractions from an anion exchange purification of a 1:1 mixture of α:β complex. Fits of the NrdE ($r^2 = 0.97$) and NrdF ($r^2 = 0.98$) standard curves were obtained. The results of this analysis indicated that NrdE and NrdF are present in a 1:1 ratio.

SI-Figure 5. SV-AUC results for Mn$^{III2}$-Y• NrdF. (A) Sedimentation coefficient distributions show two peaks whose ratio is maintained over the entire concentration range examined. The peak (~4.5 S) appears to decrease in $s_{20,w}$ as the concentration increases, suggesting it behaves non-ideally in solution. This is more clearly seen in the weight averaged $s_{20,w}$ isotherm (B), which shows a slightly decreasing slope over the concentration range tested. (C) The frictional ratio appears to remain constant over the concentration range.
SI-Figure 6. SV-AUC for NrdE in the absence of nucleotides. (A) Sedimentation coefficient distributions resulting from the $A_{280}$ data sets (SI-Table 3). The protein exists mainly as a monomer, but self-associates to produce a second species consistent with a dimer at concentrations as low as 4 µM. (B) Sedimentation coefficient distributions resulting from the $A_{250}$ data sets (SI-Table 3). Additional self-association becomes evident when $\alpha$ exceeds ~25 µM. (C) Weight averaged $s_{20,w}$ isotherm constructed from all data sets at both detection wavelengths. In the concentration ranges tested, NrdE produces no stable complexes, as evidenced by the absence of an upper asymptote. (D) The weight average $f/f_o$ decreases as NrdE concentration increases, falling below 1 at about 6 µM.
SI-Figure 7. SV-AUC results for a 1:1 mixture of NrdE:Fe$^{III}$-Y• NrdF in the absence of any nucleotides. (A) Sedimentation coefficient distributions from the $A_{230}$ data sets. Inset: a blow up of the three lowest concentration distributions. These results show an initial peak at roughly 5.5 S that broadens out and develops into a reaction boundary consisting of a mixture of low (~6.5 S) and high (~9.4 S) molecular weight species. Both peaks shift in position as the concentration increases. Also present is a peak corresponding to NrdF monomer (~3.9 S) as expected from the results of Mn$^{III}$-Y• NrdF run alone (Figure SI-6). (B) Sedimentation coefficient distributions from the $A_{280}$ data sets. The low and high molecular weight species merge forming one very broad peak that continues to change position as the protein concentration increases (~11.2 S at 3.6 µM subunits). (C) Sedimentation coefficient distributions from the $A_{250}$ data sets. New, larger molecular weight oligomeric form(s) (~12.5 S) are observed as the protein concentration increases above 5.3 µM. The peak corresponding to this species begins to separate from a peak at ~10 S as the concentration increases to ~7 µM. These data illustrate that complexes of the *B. subtilis* class Ia RNR form fairly easily, but are interconverting, despite the “tight” affinity measure by activity assays.
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