Streptococcus sanguinis Class Ib Ribonucleotide Reductase

HIGH ACTIVITY WITH BOTH IRON AND MANGANESE COFACTORS AND STRUCTURAL INSIGHTS

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Background: Class Ib ribonucleotide reductase (RNR) is an essential enzyme for aerobic growth of S. sanguinis. Results: Its manganese form is 3.5-fold more active than the iron form when assayed with NrdH and thioredoxin reductase. Conclusion: This specific activity is the highest reported to date for the class Ib RNR. Significance: Our studies suggest why manganese is important in streptococcal pathogenesis.

Streptococcus sanguinis is a causative agent of infective endocarditis. Deletion of SsaB, a manganese transporter, drastically reduces S. sanguinis virulence. Many pathogenic organisms require class Ib ribonucleotide reductase (RNR) to catalyze the conversion of nucleotides to deoxynucleotides under aerobic conditions, and recent studies demonstrate that this enzyme uses a dimanganese-tyrosyl radical (MnIII-Y) cofactor in vivo. The proteins required for S. sanguinis ribonucleotide reduction (NrdE and NrdF, α and β subunits of RNR; NrdH and TrxR, a glutaredoxin-like thioredoxin and a thioredoxin reductase; and NrdI, a flavodoxin essential for assembly of the RNR metallocofactor) have been identified and characterized. Apo-NrdF with FeII and O2 can self-assemble a diferric-tyrosyl radical (FeIII-Y) cofactor (1.2 Y/β2) and with the help of NrdI can assemble a MnIII-Y cofactor (0.9 Y/β2). The activity of RNR with its endogenous reductants, NrdH and TrxR, is 5,000 and 1,500 units/mg for the Mn- and Fe-loaded NrdF, respectively. X-ray structures of S. sanguinis NrdIox and MnIII-NrdF are reported and provide a possible rationale for the weak affinity (2.9 μM) between them. These streptococcal proteins form a structurally distinct subclass relative to other Ib proteins with unique features likely important in cluster assembly, including a long and negatively charged loop near the NrdI flavin and a bulky residue (Thr) at a constriction in the oxidant channel to the NrdI interface. These studies set the stage for identifying the active form of S. sanguinis class Ib RNR in an animal model for infective endocarditis and establishing whether the manganese requirement for pathogenesis is associated with RNR.

Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides, providing the monomeric precursors required for DNA replication and repair in all organisms (1). All RNRs rely on a metallo-cofactor to oxidize a conserved cysteine in the active site of the enzyme into a thyl radical, which then initiates reduction of nucleotides (2). In the case of the class I RNRs, two subunits are required, The β subunit contains a dinuclear metallo-cofactor that oxidizes this cysteine in the α subunit where nucleotide reduction occurs. The oxidation is reversible and occurs over a 35-Å distance (3). Recently, two new cofactors of the class I RNRs have been characterized, which has led to their subclassification (Ia, Ib, and Ic). The class Ia RNRs use a FeIII-Y cofactor in vitro and in vivo. In contrast, the class Ib RNRs are active with both MnIII-Y and FeIII-Y (4–8) cofactors, although the class Ic RNRs require a MnIVFeII cofactor (9).

In most organisms, the genes for the class Ib RNRs are found in operons. The subunits for these RNRs, α and β, are designated NrdE and NrdF, respectively. Two additional gene products are often found within this operon as follows: NrdI, a flavodoxin that we have recently shown behaves as a flavin oxidase (10), and NrdH, a glutaredoxin-like thioredoxin (11, 12). NrdF has been known for some time to be able to self-assemble an active FeIII-Y cofactor from apo-NrdF, FeII, and O2 in a manner analogous to the corresponding β (NrdB) in the class Ia
In this study, we report the cloning, expression, and isolation of NrdE, NrdF, NrdI paralogs (Nrdl, FmnI, and FmnG), NrdH, and two putative thioredoxin reductases TrxR1 and TrxR2. We establish in vitro that an active cofactor of *S. sanguinis* class Ib RNR can be assembled with both manganese and iron and that only NrdI, not FmnI or FmnG, is essential in Mn^{III}_{2-Y} cofactor formation in NrdF. The Mn^{III}_{2-Y}-NrdF has activity of 6.1 s⁻¹, 3.5-fold higher than for the Fe^{III}_{2-Y}-NrdF when assayed with NrdH and TrxR1. The Mn^{III}_{2-Y}-NrdF turnover number is very similar to the *E. coli* class Ia RNR (Fe^{III}_{2-Y}) (29). Finally, an animal model for infective endocarditis is available. Data in the accompanying paper (30) show that in vivo only the manganese NrdF appears to be active. Because mammalian host organisms use a Fe^{III}_{2-Y} for RNR activity, finding an inhibitor of NrdI/NrdF interactions required for Mn^{III}_{2-Y} cluster assembly could provide a new target for therapeutic intervention.

**MATERIALS AND METHODS**

**General**—All chemical reagents were purchased from Sigma, unless otherwise indicated. FMN was 73–79% FMN, <6% free riboflavin, <6% riboflavin diphosphates. FAD was 94% pure. MnCl₂·4H₂O (>98% pure, <5 ppm iron) and (NH₄)₂Fe(SO₄)₂·6H₂O (99%) were used as a source of Mn^{II} and Fe^{III}, respectively. Primers were synthesized by Integrated DNA Technologies. All restriction enzymes were from New England Biolabs. His₆-tagged SUMO protease was expressed in codon + Rosetta cells (Novagen) from pTBL45 provided by Prof. Bradley Pentelute (Chemistry Department, Massachusetts Institute of Technology). Buffers in this study are as follows: buffer A, 50 mM Hepes, pH 7.6; buffer B, buffer A and 5 mM β-mercaptoethanol; buffer C, 50 mM sodium phosphate, 10% (v/v) glycerol, pH 7.6; buffer D, 50 mM Hepes, pH 7.6, 15 mM MgSO₄, 1 mM EDTA. O₂-saturated buffer A was prepared immediately prior to use by sparging on ice with O₂ for at least 30 min. [³H]CDP was obtained from ViTrax and diluted with CDP in buffer A to 6,000–12,000 cpm/nmol before use. Radioactive samples were analyzed using a Beckman Coulter LS6500 scintillation counter. EPR spectra were acquired on a Bruker EMX X-band spectrometer at 77 K using a finger Dewar filled with liquid nitrogen and 706-PQ Wilmad EPR tubes or at room temperature (RT) using a Wilmad flat cell (150 μl). UV-visible spectra of anaerobic samples were acquired on a Varian Cary 3 UV-visible spectrophotometer using anaerobic cuvettes (Starna) with Teflon silicon septa (Thermo Scientific) and anaerobic titrations used a 50-μl gas tight syringe with a repeat dispenser (Hamilton). Fluorescence measurements were carried out using a Photon Technology International QM-4-SE spectrofluorometer and FELIX software. Manganese concentrations were measured using a PerkinElmer Life Sciences AAnalyst 600 atomic absorption spectrometer, and Fe₆⁴ concentrations were measured by the ferrozine assay (31). DNA sequencing and MALDI-TOF mass spectrometry were performed at the Biopolymers Laboratory, Massachusetts Institute of Technology. All anaerobic procedures were carried out in a glove box (MBraun), and all proteins were purified at 4 °C. For each protein molecular mass and the following extinction coefficients (ε) were calculated using ExPASy: NrdF or β₃ (ε₂₈₀ = 133,620 M⁻¹ cm⁻¹); NrdE or α₂ (ε₂₈₀ = 161,140 M⁻¹ cm⁻¹);
NrdH ($\varepsilon_{280} = 2,980 \text{ M}^{-1} \text{ cm}^{-1}$), Concentrations of NrdF and NrdE are reported for dimers. Concentration of NrdI$_{ox}$ was determined based on $A_{451}$ of FMN$_{ox}$ cofactor ($\varepsilon_{451} = 12,170 \text{ M}^{-1} \text{ cm}^{-1}$). Concentrations of TrxR1 and TrxR2 were estimated based on absorbance of bound FAD at 455 and 463 nm, respectively. As an extinction coefficient at these wavelengths, we used a number previously measured for $E. coli$ TrxR, $\varepsilon_{456} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$ (32).

Cloning of the $S. sanguinis$ Genes Required for RNR Activity—
Platinum Pfx DNA polymerase (Invitrogen), sense and antisense primers (supplemental Table S1) containing restriction sites (underlined), were used to amplify nrdE, nrdF, nrdI (SSA_2263), fmnG (SSA_1668), fmnI (SSA_1683), trxR2 (SSA_0813) and trxR1 (SSA_1865) from WT $S. sanguinis$ SK36 genomic DNA as a template. Taq DNA polymerase (Promega), sense and antisense primers without restriction sites, was used to amplify nrdH. All PCRs were performed using 60 ng of genomic DNA, 0.25 µg of each primer in a total volume of 50 µl, and a thermocycler program that was optimized for long AT-rich primers (supplemental Table S2). nrdE, nrdF, nrdI, fmnG, fmnI, trxR1, and trxR2 were cloned into pET28a (Novagen) and nrdF into pET24a via NdeI and BamHI or NdeI and XhoI restriction sites using T4 DNA ligase (Promega). nrdH was cloned into pETSUMO using T4 DNA ligase as described in the manufacturer’s protocol (Invitrogen).

Expression and Purification of NrdH, FmnG, FmnI, Apo-NrdF, and NrdE—pET28a-nrdH was transformed into BL21(DE3) cells and expressed in LB in the presence of 50 µg/ml kanamycin. The culture was grown at 37 °C with shaking at 200 rpm to OD$_{600}$ = 0.5, and then riboflavin was added to a final concentration of 10 mM. NrdH culture was induced at 0.5, and then riboflavin was added to a final concentration of 0.4 mM and incubated at 30 °C. In 4–5 h, cells were harvested by centrifugation at 3,000 g for 10 min at 4 °C, frozen in liquid nitrogen, and stored at −80 °C. Typical yield was 2.3 g of cell paste/liter of culture.

Cell pellet (9.2 g) was resuspended in 46 ml of buffer B containing one Complete protease inhibitor mixture tablet. The cell suspension was passed twice through a French pressure cell at 14,000 p.s.i. and then centrifuged at 20,000 × g for 25 min. Nucleic acids were precipitated by the addition of a streptomycin sulfate solution to a final concentration of 0.5% (w/v) with stirring over 20 min. After the solution was spun down at 20,000 × g for 30 min, the supernatant was loaded onto Ni-NTA-agarose column (1.5 × 2.5 cm, 4.4 ml) pre-equilibrated with buffer B containing 30 mM imidazole, and the column was washed with the same buffer until $A_{260}$ of the flow-through was <0.02. The protein was eluted with 50 ml of 200 mM imidazole in buffer B, and 2-ml fractions were collected. Fractions containing SUMO-NrdH (21.6 kDa) were identified by SDS-PAGE (12% (w/v) acrylamide), pooled, and loaded onto a Q-Sepharose Fast Flow column (2.5 × 5.5 cm, 27 ml) pre-equilibrated with 100 mM NaCl in buffer B. The column was then washed with the same buffer (300 ml) then SUMO-NrdH was eluted with 100 × 100-mL linear gradient of 100–500 mM NaCl in buffer B, and 3.6-ml fractions were collected. Fractions with high $A_{280}$ were analyzed by SDS-PAGE, pooled, and concentrated to 470 µM using an Amicon YM-10 filter.

SUMO-NrdH (470 µM) was divided into 0.6-ml aliquots, and each was incubated with SUMO protease (150 µL, 50 µM) overnight at 4 °C. About 60% of the protein was cleaved under these optimized conditions. The digested SUMO-NrdH was loaded directly onto a Ni-NTA-agarose column (1.5 × 2.5 cm, 4.4 ml) pre-equilibrated with 30 mM imidazole in buffer B. The column was washed with the same buffer, and 1-ml fractions were collected. Fractions containing NrdH (8.2 kDa), assessed by SDS-PAGE analysis (16% (w/v) Tricine gel, Invitrogen), were pooled and concentrated using an Amicon YM-3 filter. NrdH (2 ml) was then loaded onto Sephadex G-25 column (1.5 × 33 cm, 58 ml) pre-equilibrated with buffer A containing 10 mM DTT and 1 mM EDTA, eluted with the same buffer, and concentrated to 1 mM using an Amicon YM-3 filter. The ratio $A_{280}/A_{260}$ of homogeneous NrdH is 1.3. Successful removal of the tag was confirmed by MALDI-TOF MS.
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To remove DTT from NrdH, required for the DTNB assay, NrdH (400 µl, 1 mM) was loaded onto Sephacry G-25 column (1 x 6.5 cm, 5 ml) pre-equilibrated with buffer A, and 0.5-ml fractions were collected. Fractions containing NrdH, as judged by A_{280}/A_{260}, were pooled and concentrated to 450 µM as described above.

pET28a-trxR1 (or pET28a-trxR2) was transformed into BL21(DE3) cells and trxR1 (trxR2) overexpressed in the presence of 50 µg/ml kanamycin. The culture was grown at 37 °C to OD_{600} = 0.5, and then riboflavin (10 mg/liter) was added, and the temperature was lowered to 30 °C; 10 min later, the culture was induced with isopropyl-β-d-1-thiogalactopyranoside to a final concentration of 0.4 mM. In 4 h, the cells were harvested, as above to give typical yields of 2.5 g wet cell paste/liter of culture.

Cell pellet (7.5 g) was resuspended in 38 ml of buffer A containing a Complete protease inhibitor tablet and 1.7 mM FAD. The cell suspension was passed twice through a French pressure cell at 14,000 p.s.i. and then centrifuged at 20,000 x g for 25 min. Nucleic acids were precipitated by the addition of streptomycin sulfate to a final concentration of 1% (w/v). After the solution was spun down at 20,000 x g for 30 min, the supernatant was loaded onto a Ni-NTA-agarose column (1 x 4.2 cm, 3 ml), pre-equilibrated with buffer A with 30 mM imidazole, and the column was washed with the same buffer until A_{280} was <0.02. The protein was eluted with 20 ml of 200 mM imidazole in buffer A. The yellow fractions were pooled, diluted 4-fold with buffer A, and loaded onto Q-Sepharose Fast Flow column (2.5 x 5.5 cm, 27 ml), pre-equilibrated with buffer A and 100 mM NaCl, and the column was washed with the same buffer (~150 ml). TrxR1 (TrxR2) was eluted with 100 x 100-ml linear gradient of 100-550 mM NaCl in buffer A and concentrated using an Amicon YM-30 filter; NaCl was removed by further dilution/concentration in buffer A.

Optimized Cofactor Assembly from Apo-NrdF Loaded with Fe^{II} or Mn^{II}—Buffers and proteins were degassed on a Schlenk line with at least five cycles of evacuation and refilling with argon. Concentrated NrdF_{ox} (0.5–1 mM) and apo-NrdF (0.8 mM) were stable at 4 °C for at least 5 days. To prepare Mn^{II}-NrdF, apo-NrdF (45 µl, 800 µM) was incubated with 6 eq of Mn^{II} (2 mM solution, freshly prepared) in buffer A at 37 °C for 15 min. A mixture of Mn^{II}-NrdF (60 µM) and NrdF_{ox} (120 µM) in buffer A in a total volume of 600 µl was titrated anaerobically with dithionite (~3 mM, standardized using potassium ferricyanide (33)) until NrdF_{ox} was completely reduced to NrdF_{hq} (judged by the disappearance of the band at 580 nm associated with NrdF_{ox}). Cluster assembly was initiated by bubbling O_{2} through the Mn^{II}-NrdF/NrdF_{hq} solution for ~1 min. This protocol typically gave 0.6 Y/β_{2}. Increased yields of Y/β_{2} were obtained by recycling NrdI. After cluster assembly as described above, the mixture of Mn^{II}-Y (0.6 Y/β_{2}) and NrdF_{ox} was again degassed on a Schlenck line (six cycles) and transferred inside the glove box to an anaerobic cuvette. The UV-visible spectrum was recorded to ensure that radical content remained intact, and then the mixture was titrated with dithionite (~3 mM) to reduce NrdF_{ox} to NrdF_{hq}. O_{2} was then bubbled through the sample for ~1 min. To remove free Mn^{II} and NrdI, the mixture was incubated with EDTA (5 mM) at 4 °C for ~30 min and loaded onto a Q-Sepharose Fast Flow column (1 x 3 cm, 2.5 ml) pre-equilibrated with 200 mM NaCl in buffer A. The column was washed with 200 mM NaCl (buffer A, 25 ml), and Mn^{III}-Y-NrdF was eluted with 500 mM NaCl (buffer A, 20 ml); 1-ml fractions were collected, and the protein was concentrated using an Amicon YM-30 filter. Typical radical content was 0.9–1.0 Y/β_{2}.

For cluster assembly with iron, apo-NrdF (~150 µl, 900 µM) was incubated anaerobically with 6 eq of Fe^{III} at 37 °C for 15 min and then diluted with buffer A to 60 µM. Cluster assembly was initiated by addition of an equal volume of oxygenated buffer A. To remove excess iron, ferrozine (80-fold excess over NrdF) and sodium dithionite (40-fold excess) were added, and the mixture was incubated on ice for 5 min. The protein was then desalted on a Sephadex G-25 column (1 x 11 cm, 8.5 ml) in buffer A. NrdF-containing fractions were pooled and concentrated. The amount of NrdF-bound Fe^{III} was measured by the ferrozine assay (31).

Y Quantitation—All EPR spectra used for spin quantitation were acquired under nonsaturating conditions at 77 K (4). Spin quantitation was performed by double integration of the signal and comparison with a standard of E. coli Fe^{III}-Y-NrdB.

K_{d} for the NrdF_{hq} and Mn^{II}-NrdF Interaction—The procedure is a minor modification of that previously reported (16). Under anaerobic conditions at 23 °C, NrdF_{hq} (240 or 360 µM, ~ 40 µl) in buffer C was added in 2-µl portions using an air-tight Hamilton syringe into Mn^{III}2-NrdF (1 or 3 µM, 700 µl) in the same buffer. To ensure that NrdF_{hq} remains reduced throughout titration, buffer C also contained 100 µM dithionite. After each injection, the cuvette was inverted several times, and the solution was allowed to equilibrate at RT in the dark for 1 min, and the spectrum was recorded. The excitation wavelength was 380 nm, and the emission was measured from 475 to 625 nm in 1 nm steps with a 1-s integration time. The excitation and emission bandwidth slit was 1.5 and 0.75 mm, respectively. No photobleaching was detected using these settings. Data were analyzed by the method of Eftink (34). The titration was performed four times using different concentrations of Mn^{III}2-NrdF and NrdF_{hq} and the data were fit in IgorPro to obtain the stoichiometry of NrdF_{hq} binding to NrdF (n) and the dissociation constant (K_{d}).

General Crystallographic Methods—All crystallographic datasets were collected at the Life Sciences Collaborative Access Team (LS-CAT) or General Medical Sciences and Cancer Institutes Collaborative Access Team (GM/CAT-CAAT) beamlines at the Advanced Photon Source and processed using the HKL2000 software package (35). Iterative rounds of refinement and model building were performed using Refmac5 (36) and Coot (37). Ramachandran plots were calculated with Molprobity (38) and figures were generated with the PyMOL Molecular Graphics System (Schrödinger, LLC). Internal channel calculations were performed with HOLLOW (39) using a 1.4 Å probe radius. Electrostatic surface potential calculations were carried out using the PyMOL APBS plugin (40). Electron density maps were calculated with FFT (41). Table 1 reports all data collection and refinement statistics.

X-ray Structure Determination of S. sanguinis Mn^{II}-NrdF—Crystals of S. sanguinis Mn^{II}-NrdF (25 mg/ml in 20 mM Hepes buffer, 5% (v/v) glycerol, pH 7.6) were generated using the...
hanging drop vapor diffusion method with 25% (w/v) PEG 3000, 250 mM magnesium formate, and 100 mM Hepes, pH 7.6, as the precipitating solution. Crystals appeared after 2 weeks of incubation at RT and were prepared for data collection by mounting on rayon loops and flash freezing in liquid nitrogen following cryoprotection by brief soaking in well solution containing 25% (v/v) glycerol. X-ray diffraction datasets were processed as described above with additional scaling performed using the UCLA MBI Diffraction Anisotropy Server (42). The structure was solved by molecular replacement using BALBES (43) with the Salmonella typhimurium Fe(III)-NrdF structure (PDB accession code 1R2F) as the search model. Eight copies of NrdF, arranged into four β2 dimers, are present in the asymmetric unit. The quality of the electron density map varies widely between the eight monomers. The electron density is the least well defined for two of the monomers (chains A and G) and is of the highest quality for chains B and H. The latter subunits were used to draw conclusions about the structural features of the metal-binding site and oxidant channel. To aid in model building, tight noncrystallographic symmetry restraints were used in the initial phases of model refinement and released in the final rounds. The final model consists of residues 3–287 for chain A, residues 3–286 for chains B–F, residues 4–286 for chains G and H, two MnII ions per NrdF monomer, and 64 water molecules. Ramachandran plots show that 99.9% of residues are in allowed and generously allowed regions.

X-ray Structure Determination of S. sanguinis NrdIox—Crystals of S. sanguinis NrdIox (25 mg/ml in 20 mM Hepes buffer, 5% (v/v) glycerol, pH 7.6) were generated from a commercial screen (Qiagen) using the sitting drop vapor diffusion method with 30% (w/v) PEG 4000, 200 mM ammonium sulfate, and 100 mM sodium acetate, pH 5.6 as the precipitating solution. Crystals appeared after 1 week of incubation at RT and were prepared for data collection by addition of a well solution, in a 1:1 ratio, containing 50% (v/v) glycerol to the crystallization drop followed by mounting on rayon loops and flash freezing in liquid nitrogen. The structure was solved by molecular replacement using PHASER (44) with B. subtilis nrdI (PDB accession code 1RLJ) as the search model. Ramachandran plots indicate that 100% of residues are in allowed and generously allowed regions. The asymmetric unit contains two NrdI monomers and the final model consists of residues 2–66, 72–154 in chain A, residues 2–154 in chain B, two FMN molecules, two sulfate molecules, and 204 water molecules. Residues 67–71 in chain A are disordered and could not be modeled. Attempts to determine the structure of reduced forms of NrdI by soaking NrdIox crystals in 10–100 mM solutions of dithionite produced color changes in the crystals, but structures obtained from the resulting diffraction datasets did not exhibit any significant conformational changes in response to FMN reduction.

DTNB Assay for TrxR1/TrxR2—In a final volume of 290 μl NADPH (300 μM), variable amounts of DTT-free NrdH (0.06–5 μM), 100 mM Tris (pH 7.5 at 20 °C), and 2 mM EDTA were mixed. DTNB was added to a final concentration of 1 mM, and the mixture was equilibrated to 25 °C in a cuvette. The reaction was initiated by addition of TrxR1 (17.5 nM/dimer) and monitored by change in A412. The turnover number was calculated as described previously (45). A similar experiment was carried out with TrxR2 (17.5 nM/dimer) and NrdH (5–30 μM); no change in A412 was observed.

Activity Assays—Three sets of conditions optimized to assay S. sanguinis RNR using DTT, NrdH/DTT, or NrdH/TrxR1/NADPH as the reductant are described. 1) For DTT a typical activity assay contained in a final volume of 170 μl the following: reconstituted NrdF (0.2 μM), NrdE (2 μM), dATP (100 μM), DTT (20 mM), [3H]CDP (0.5 μM, 6,644 cpm/nmol) in buffer D at 37 °C. Aliquots (30 μl) were removed at 0, 3, 6, 9, and 12 min, and the reaction was stopped by heating at 100 °C for 2 min. 2) For NrdH/DTT, a typical assay contained in a final volume of 170 μl the following: NrdF (0.07 μM), NrdE (0.14 μM), dATP (100 μM), DTT (20 mM), NrdH (10 μM), [3H]CDP (0.5 μM, 6,644 cpm/nmol) in buffer D at 37 °C, and aliquots (30 μl) were taken at 0, 1, 2, 3, 4 min. 3) For NrdH/TrxR1/NADPH, a typical assay contained in a final volume of 170 μl the following: NrdF (0.07 μM), NrdE (0.14 μM), dATP (100 μM), NrdH (DTT-free, 10 μM), TrxR1 (0.5 μM), NADPH (1 mM), [3H]CDP (0.5 μM, 6,644 cpm/nmol) in buffer D at 37 °C, and aliquots (30 μl) were taken at 0, 1, 2, 3, 4 min. [3H]dCDP was quantitated by the method of Steepe and Steuert (46). One unit of activity is defined as the production of 1 nmol of dCDP per min.

Km for NrdE–NrdF Interaction (47)—The reactions contained in a final volume of 170 μl the following: dATP (100 μM), DTT (20 mM), NrdH (10 μM), [3H]CDP (0.5 μM), and NrdF/NrdE in a ratio 1:1 (1 to 80 nM) in buffer D at 37 °C. Protein solutions below 0.1 μM also contained BSA (0.2 mg/ml). Aliquots (30 μl) were taken over 12 min for samples containing 1–5 nM, over 8 min for samples with 7–10 nM, and over 4 min for samples containing 20–80 nM NrdF and NrdE.

RESULTS

Identification of the Genes for Cluster Assembly and Activity of S. sanguinis RNR—The genome of S. sanguinis SK36 has been sequenced, and nrdH-nrdE-nrdK-nrdF were annotated in a single operon (48). A search for nrdl using B. subtilis nrdl as the query sequence revealed three candidate genes: SSA_2263 (nrdl), SSA_1668 (fmnG), and SSA_1683 (fmnl) (Fig. 2A). A general screen for genes essential under aerobic conditions identified only SSA_2263 (49). Thus, SSA_2263 was tentatively annotated as NrdL. Further analysis of this nrdl sequence, specifically the spacing between the ribosomal binding site and start codon, and ClustalW sequence alignments of characterized NrdLs suggested that Met5 is the actual start site and that the annotated start site is incorrect (Fig. 2B and supplemental Fig. S1). SSA_1668 and SSA_1683 were shown to bind FMN, and their genes were named fmnG and fmnL, respectively. Finally, to identify candidates for TrxR, B. anthracis thiorodoxin reductase (BA5387) was chosen for a BLAST search, and two candidate genes, SSA_1865 or trxR1 (TrxR1) and SSA_0813 or trxR2 (TrxR2), were identified. Studies in the accompanying paper (30) reveal that only SSA_1865 is essential under aerobic growth conditions.

Expression and Purification of Apo-NrdE, NrdE, NrdF, FmnG, FmnL, NrdH, and TrxR5—Genes encoding NrdE, NrdF, NrdL, FmnG, NrdH, and TrxR5 were amplified by PCR using S. sanguinis SK36 genomic DNA as a template. The gene
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for each protein was cloned into pET vectors for expression in BL21(DE3), and the sequences were verified. Apo-NrdF was expressed without a tag in pET24a. NrdE, Nrdg, FmnI, FmnG, TrxR1, and TrxR2 all were expressed in pET28a with an N-terminal His6 tag and a 10-amino acid linker (MGSSHHHH-HHSSGLVPRSGSH). To obtain high yields of soluble protein, NrdH was cloned into pETSUMO and expressed as a fusion with a His6-SUMO tag, and subsequent to protein purification, the His6-SUMO tag was removed using SUMO protease. All of the proteins were purified to >95% homogeneity by conventional methods (Ni-NTA and ion exchange chromatography).

Characterization of NrdI—Phylogenetic analysis suggests that S. sanguinis NrdI is likely distinct from previously characterized NrdIs (5, 10, 14, 16). Thus, NrdI (full-length and truncated by four amino acids, Fig. 2A) along with FmnI and FmnG were expressed and purified as discussed subsequently, neither FmnI nor FmnG support MnIII in NrdF, whereas both the full-length and truncated NrdIs support MnIII in NrdF. This gives a further indication that the truncated NrdI was used in subsequent experiments.

The characterization of S. sanguinis NrdI followed our recent protocols for B. cereus NrdE and the RNR operon containing the genes for the redoxin that re-reduces the active site disulfide (nrdF), the two subunits of RNR (nrdE and nrdF), and an unknown open reading frame nrdK (5, 10) for two possible start codons for NrdI. Sequences upstream of the putative nrdI start codons are in black, the start codons and nrdI are in red, RBS, ribosomal binding site.

Typically in cluster assembly studies, MnIII-Yapo-NrdF was made aerobic with MnIII (6 eq), incubated at 37 °C for 15 min, and the free MnII was removed by Sephadex G-25 chromatography. Atomic absorption analysis typically gave 3.6–3.8 MnIII per β2. The EPR spectrum of MnIII-NrdF at 9 K (Fig. 3C) is very similar to E. coli and S. typhimurium MnIII-NrdFs and distinct from the B. subtilis MnIII-NrdF (4, 14, 16).

MnIV-Yapo-NrdF was washed and anaerobic and titrated with dithionite to reduce MnIII. The UV-visible spectrum of the resulting NrdF (Fig. 3D, line 2) reveals a typical Y feature at 408 nm and absorption features at 550 and 330 nm associated with the MnIV cluster. Quantitative analysis typically gives 0.5–0.6 Y/β2 and 3.1–3.4 MnIV/β2. Many variables (pH, temperature, and ratio of manganese/NrdF) were examined in an effort to increase the amount of Y/β2, all conditions resulted in similar yields. A higher yield of Y/β2, however, was achieved by recycling the NrdI. The NrdI recycle mixture resulting from the first effort to assemble cluster (Fig. 3D, line 2) was made aerobic and titrated with dithionite to reduce NrdIox into NrdIeq. The sample was then re-exposed to O2 (Fig. 3D, line 3). This recycling process typically gives 0.9 Y/β2 and 3.7 MnIV/β2. The EPR spectrum of the MnIV-Y response at 77 K is a broad signal with a line width of 150 G and is shown in Fig. 3B (black line). Furthermore, as in the case of the other MnIV-Y-NrdF (4, 5, 22), the spectrum dramatically sharpens at 4 K (data not shown). Attempts to assemble MnIV-Y cluster using FmnIeq or FmnGeq and conditions optimized for NrdI were unsuccessful.

The increase in active MnIV-Y-NrdF cluster by NrdI recycling likely mimics the assembly process in vivo where a flavodoxin reductase would recycle catalytic amounts of NrdF (5, 23). However, a search of the S. sanguinis genome failed to reveal any candidate homologous to E. coli flavodoxin reductase (FdxR).

Quantitative Characterization of the Association of NrdIeq with MnIV-NrdF—To assess the affinity of NrdIeq for MnIV-NrdF, we took advantage of the previous observation in E. coli and B. subtilis systems that NrdIeq fluorescence is altered by the presence of NrdF (16). Similarly, fluorescence of S. sanguinis NrdIeq increases 8-fold in the presence of S. sanguinis MnIV-Y...
NrdF. Thus, titration studies were carried out as described previously (16), and the analysis reveals a \( K_d \) of 2.9 \( \pm \) 1 \( \mu \)M with 1.5 \( \pm \) 0.4 NrdIhq per NrdF. This \( K_d \) value is higher than those we previously reported for the NrdII-NrdF interactions in \( E. coli \) (\( K_d < 0.05 \mu \)M) and \( B. subtilis \) (\( K_d = 0.6 \mu \)M) (16). Given the \( K_d \) value and the concentrations of Mn\( ^{112} \)-NrdF (60 \( \mu \)M) and NrdIhq (120 \( \mu \)M) used to study cluster assembly described above, 95% of Mn\( ^{112} \)-NrdF is complexed.

To further explore the similarities/differences among NrdFs from various organisms, an additional experiment was carried out. Previous studies in the \( E. coli \) system showed that reduction of NrdIox in the presence of apo-NrdF (1 eq) led to formation of an anionic FMN semiquinone (~30%). In a similar experiment with \( B. subtilis \) NrdF, no anionic form was detected (16). We evaluated the FMN form that accumulated upon reduction of \( S. sanguinis \) NrdI in the presence of apo-NrdF, and no anionic semiquinone flavin was observed.

**Crystallographic Analysis of the \( S. sanguinis \) Mn\( ^{112} \)-NrdF—**
Because the streptococcal NrdFs are in a phylogenetic group that has not previously characterized, we determined the structure Mn\( ^{112} \)-NrdF (2.65 \( \AA \) resolution, Table 1) to compare with the corresponding structures of the \( E. coli \) and \( B. subtilis \) NrdFs. As shown in Fig. 4, the Mn\( ^{112} \)-binding site strongly resembles that of the \( E. coli \) Mn\( ^{112} \)-NrdF structure (15). Each Mn\( ^{112} \) ion is six-coordinate with three bridging carboxylates, including the unusual bridging mode for Glu\( ^{122} \) (\( S. sanguinis \) numbering), two His ligands, and two coordinated water molecules. The side chain of Asp\( ^{56} \) hydrogen bonds to Tyr\( ^{104} \), the site of \( Y^* \) formation in the active metallo-cofactor, and also resembles the linkage between the corresponding Tyr residue and the metal-binding site in \( E. coli \) NrdF. Although the moderate resolution prevents conclusive determination of the orientation of Glu\( ^{122} \), the residue is modeled in a \( \mu - \eta^1, \eta^2 \) binding mode, as observed in \( E. coli \) Mn\( ^{112} \)-NrdF, and the refined model is consistent with this assignment. The structural similarity to \( E. coli \) Mn\( ^{112} \)-NrdF is consistent with similarities observed in the EPR spectra of these proteins.

The unusual configuration of the carboxylate side chains in the \( E. coli \) Mn\( ^{112} \)-NrdF structure opens a solvent-lined channel near Mn2 to the surface of protein (Fig. 5A). The channel is further accommodated by small hydrophobic residues near

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**FIGURE 3. Spectroscopic characterization of \( S. sanguinis \) NrdI, Mn\( ^{112} \)-NrdF, and assembled clusters.**

A, spectra of NrdIox (red), NrdIhq (black), and NrdIox-5,000 (blue) in the presence (dotted) or absence (solid) of NrdF in buffer C. B, comparison of the EPR spectra (77 K) of \( S. sanguinis \) Fe\( ^{112}\)-Y-NrdF (1.2 \( \gamma^1 \)/\( \beta^2 \), acquired at 50 microwatt power), and Mn\( ^{112}\)-Y-NrdF (0.9 \( \gamma^1 \)/\( \beta^2 \), 1 milliwatt power). Spectra were normalized for NrdF concentration and power. Other spectrometer settings were 9.45 GHz, 1.5 G modulation amplitude, and 10.24 ms time constant. C, EPR spectrum of Mn\( ^{112} \)-NrdF (380 \( \mu \)M) at 9 K, 0.1 milliwatt. To prepare Mn\( ^{112} \)-NrdF, apo-NrdF (210 \( \mu \)l, 900 \( \mu \)M) was incubated with 6 eq of Mn\( ^{112} \) in buffer A at 37 °C for 15 min. Unbound Mn\( ^{112} \) was removed using a Sephadex G-25 column (36.5 \( \times \) 1.5 cm, 64 ml) in buffer A, and the protein was concentrated on an Amicon YM-30 filter. The resulting protein contained 3.8 Mn\( ^{112} \)-NrdF (210 \( \mu \)l, 900 \( \mu \)M) (16). Given the 95% of Mn\( ^{112} \)-NrdF is complexed.

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**TABLE 1**

**Data collection and refinement statistics for the \( S. sanguinis \) Mn\( ^{112} \)-NrdF and NrdIox x-ray structures**

|                      | \( S. sanguinis \) Mn\( ^{112} \)-NrdF | \( S. sanguinis \) NrdIox |
|----------------------|----------------------------------------|--------------------------|
| **Space group**      | \( P2_1 \)                              | \( C2 \)                  |
| **Resolution**       | 2.65 \( \AA \) (2.70 to 2.65 \( \AA \)) | 1.88 \( \AA \) (1.91 to 1.88 \( \AA \)) |
| **R\( _{work} \)**   | 0.166 (0.747)                           | 0.084 (0.705)            |
| **Completeness**     | 108 (2.2)                               | 16.9 (2.4)               |
| **Bond angles**      | 0.237 (0.276)                           | 0.207 (0.238)            |
| **Average B-factor**| 28.7                                   | 24.2                     |
| **Root mean square deviations** | 0.006 \( \AA \) | 0.005 \( \AA \) |
| **Bond angles**      | 0.849 (1.008)                           | 0.849 (1.008)            |

*Values in parentheses refer to the highest resolution shell.

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For calculation of R\( _{free} \) 5% of the reflections was reserved.
Mn2 (Ala75 and Ile94 in *E. coli* NrdF (53)). *B. subtilis* MnII

2-NrdF, which has a different MnII coordination environment and larger Met residues in place of the Ala/Ile pair adjacent to the Mn2 site, does not contain a solvent-occupied channel (53). Because *S. sanguinis* MnII

2-NrdF shares a similar coordination environment with *E. coli* MnII

2-NrdF and retains the hydrophobic residues near Mn2 (Val74 and Ile93), it is not surprising that it contains a similar channel (Fig. 5 B). The overall cavity shape and volume are nearly identical to the *E. coli* NrdF channel, and electron density for water molecules is also observed (Fig. 5, C and D). This solvent-accessible channel is modeled as a cyan transparent surface. C, 2F_o − F_c electron density map (cyan mesh) contoured at 1.2σ reveals ordered water molecules in the channel in the chain B NrdF monomer. D, zoomed-in view of the modeled waters near the Mn2 site and associated hydrogen-bonding interactions. Selected amino acid side chains and backbone atoms are shown in stick format. MnII ions (magenta) and water molecules (cyan) are shown as spheres.

Another interesting conserved feature in the *S. sanguinis* and *E. coli* MnII

2-NrdF structures involves a constriction in the channel immediately above Mn2. In the *E. coli* system, the constriction is formed by the side chain of Ser159 and the backbone carbonyl of the bridging ligand Glu192, whereas in the *S. sanguinis* system it involves Thr158 and Glu191 (Fig. 5, A and B, supplemental Fig. S2). Because oxidant passage through this constriction may be a slow step in metallo-cofactor activation, substitution to a more sterically bulky residue could translate into a slower rate of reaction with the oxidant in the *S. sanguinis* system.

Crystal Structure of *S. sanguinis* NrdIox—NrdIs have been structurally characterized thus far from several species of *Bacillus* (50, 51) and *E. coli* (in complex with MnII

2-NrdF) (15). As noted above, streptococcal NrdIs represent a third phylogene-
The short loop (XFG) in the Bacillus Nrdls is similar to S. sanguinis Nrdl_{ox} in that neither can form this interaction. The Bacillus and E. coli Nrdls have also been structurally characterized in the sq- or hq-reduced states (15, 50, 51). All of these structures reveal a backbone amide flip that positions a carbonyl group near the N5 position to interact with the now protonated form of the cofactor.

To understand whether a similar conformational change occurs upon reduction of S. sanguinis NrdI, diffraction datasets were collected on dithionite-soaked Nrdl_{ox} crystals. This treatment reduced the FMN to the sq or hq state, based on observation of color change in the crystals, but the resulting electron density maps did not reveal any significant conformational changes in the 70s loop backbone. The protonated FMN cofactor (FMNH\textsuperscript{2+} or FMNH\textsuperscript{+}) interacts instead with a water molecule (not observed in the Nrdl_{ox} structure). The observed outcome in the dithionite-soaked S. sanguinis Nrdl crystals could be the result of crystal packing interactions that preclude conformational changes in the loop or it could indicate that the 70s loop backbone cannot, in any oxidation state, bind directly to the FMN N5 position due to the \(\beta\)-sheet hydrogen bonding pattern in the loop near the FMN (Fig. 6B).

The S. sanguinis NrdI 70s loop is also unusual in that it contains many more bulky and negatively charged residues than the corresponding regions in other Nrdls. These residues could affect the electrostatic properties of the flavin and its accessibility to \(O_2\). However, evaluation of the electrostatic environment in Nrdl near the FMN isalloxazine ring shows that the interior of the FMN pocket remains positively charged, similar to what is observed in E. coli and the Bacillus Nrdls (Fig. 7). The negatively charged residues in the S. sanguinis NrdI 70s loop instead generate a strong negative patch on the exterior of the protein. We propose that this patch may be involved in interaction with a flavin reductase required to recycle Nrdl_{ox} \textit{in vivo}.®
have measured the activity of pure reconstituted Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−} and Fe\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−} with DTT, DTT/NrdH, and the endogenous reducers NrdH and TrxR1 (Fig. 1). Some representative results of optimization of the concentrations of NrdE, NrdF, and variable reducers are shown in Fig. 8, A−C.

The $K_d$ value for NrdE-NrdF interactions in the class Ib RNRs still remains largely unknown. However, recent studies on B. subtilis class Ib RNR demonstrated that the active form is a 1:1 complex of subunits (5, 47). Thus, our initial studies focused on establishing the ratio of NrdE to NrdF for maximum activity using DTT and NrdH/DTT as reductant. First, Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−}/NrdF (0.9 Y/β\textsubscript{2}) at 0.2 μM was assayed with increasing concentrations of NrdE (0.4−4 μM) and DTT in excess. Under these conditions, maximum activity is observed at 5 eq of NrdE (Fig. 8A, inset). When the assay was carried out with Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−}/NrdF (0.07 μM) using NrdH as the reductant, the SA of Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−} was 20-fold higher relative to DTT and only 1 to 2 eq of NrdE were required for maximum activity (Fig. 8A)). Differences in the NrdE/NrdF ratios required for maximal activity with DTT and NrdH could in part reflect the higher efficiency of NrdH in recycling NrdE, which results in higher concentrations of reduced NrdE available for each turnover. In almost all subsequent assays, a 1:2 ratio of NrdF to NrdE was used.

Physiological Reductant for NrdE—The reduction of NDPs to dNDPs is accomplished by oxidation of two active site cysteines in NrdE to a disulfide (Fig. 1) (2, 55). A number of artificial and endogenous systems are capable of mediating this re-reduction step, including dithiothreitol (DTT), Trx/TrxR/NADPH, Grx/GSH/GR/NADPH, and NrdH/TrxR/NADPH, where Trx is thioredoxin, Grx is glutaredoxin, and GR is glutaredoxin reductase (6, 11, 20, 21, 56). The observation that NrdH is co-localized in the same operon with nrdE and nrdF in S. sanguinis and many other organisms makes this protein the most reasonable candidate for the endogenous reductant. Our initial attempts to isolate untagged NrdH were unsuccessful due to its low solubility. To overcome this problem, NrdH was fused to a His\textsubscript{6}-SUMO tag and then isolated by nickel-affinity chromatography. Subsequent removal of the tag with SUMO protease gave soluble NrdH, which has been used with TrxR to assay RNR. Two candidate genes (trxR1 and trxR2) for thioredoxin reductases were identified, and the corresponding proteins, TrxR1 and TrxR2, were overexpressed and purified to homogeneity by nickel-affinity chromatography. To ensure that the isolated TrxRs were fully loaded with cofactor, FAD was added to crude cell lysates prior to purification (6, 57). This protocol gave homogeneous FAD-bound TrxR1 and TrxR2 with the ratio $A_{272}/A_{455}$ of 6.0 and 6.4, respectively. Complete cofactor loading was further confirmed by anion exchange chromatography.

The turnover number for each TrxR was measured using NADPH, NrdH, and the DTNB assay (45). The $K_m$ value of TrxR1 for NrdH was 0.09 μM and $k_{cat} = 3.5$ s\textsuperscript{−}1 giving $k_{cat}/K_m$ of 4.03 × 10\textsuperscript{7} M\textsuperscript{−}1 s\textsuperscript{−}1. TrxR2, however, showed no activity under the same conditions. Thus, the results suggest that TrxR1 is the reductant for NrdH in vivo.

Activity of Fe\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−} and Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−} NrdFs Using DTT, NrdH/DTT, and NrdH/TrxR1/NADPH—The activity of the class Ib RNRs has been predominantly reported for the Fe-loaded NrdFs (14, 21, 58, 59), as the role of NrdI in generation of active Mn-loaded RNRs was not elucidated until 2010. Although a number of recent studies have reported activities for Mn\textsuperscript{III}z\textsuperscript{2+}Y\textsuperscript{−}/NrdFs, in most cases the proteins contained substoichiometric manganese loading and Y\textsuperscript{−} and/or the endogenous reductant was not used (4−6, 8). As a starting point to identify the metallo cofactor required for S. sanguinis class Ib in cultures and in an animal model for S. sanguinis-mediated endocarditis, we
S. sanguinis Class Ib Ribonucleotide Reductase

FIGURE 8. SA of MnIII$_2$Y$_2$-NrdF (0.9 Y/β$_2$). A, SA of NrdF in the presence of variable amounts of NrdE using DTT (red) or NrdH/DTT (black) as a reductant. B, SA measured with variable [NrdH], 70 nM [NrdE], [NrdF], 0.1 mM [dATP], and 20 mM [DTT]; C, $K_m$ value for the interaction between NrdF and NrdE using a 1:1 ratio of subunits, 0.1 mM [dATP], 10 μM [NrdH], and 20 mM [DTT]; D, SA measured in the presence of variable dATP concentrations, 70 nM [NrdF], 0.14 μM [NrdE], 10 μM [NrdH], and 20 mM [DTT]. NrdE, specific activity of 3,000 units/mg, was used in all experiments. All plots were fit to Michaelis-Menten equation using IgorPro.

TABLE 2
Activity of MnIII$_2$Y$_2$-NrdF and FeIII$_2$Y$_2$-NrdF reconstituted in pure proteins with different reductants

| Metal cofactor | Y'/β$_2$ | A, SA of NrdF in the presence of variable amounts of NrdE using DTT (red) or NrdH/DTT (black) as a reductant. | B, SA measured with variable [NrdH], 70 nM [NrdE], [NrdF], 0.1 mM [dATP], and 20 mM [DTT]; C, $K_m$ value for the interaction between NrdF and NrdE using a 1:1 ratio of subunits, 0.1 mM [dATP], 10 μM [NrdH], and 20 mM [DTT]; D, SA measured in the presence of variable dATP concentrations, 70 nM [NrdF], 0.14 μM [NrdE], 10 μM [NrdH], and 20 mM [DTT]. NrdE, specific activity of 3,000 units/mg, was used in all experiments. All plots were fit to Michaelis-Menten equation using IgorPro. |
|---------------|---------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|
| Mn            | 0.9     | 260 ± 30                                                             | 5,700 ± 460                                                 | 5,000 ± 175                                                 | 3.7 ± 0.2                                           |
| Fe            | 1.2     | 170 ± 30                                                             | 1,500 ± 360                                                 | 1,500 ± 100                                                 | 3 ± 0.5                                             |

Y'-NrdF was measured in the presence of increasing dATP concentrations (Fig. 8D). The apparent $K_m$ is 2.4 μM, and no inhibition was observed even at 1 mM dATP.

DISCUSSION

Since our discovery that NrdI plays an essential role in MnIII$_2$Y$_2$ formation, many reports have appeared describing the activity and properties of manganese-loaded class Ib RNRS. Although the identification of the endogenous reductant has been reported in some of the studies, in most cases the activity of the class Ib RNR remained very low even with the endogenous reductant (6, 8). We have chosen to examine the importance of manganese- versus iron-loaded cofactor in S. sanguinis class Ib RNR because this organism causes infective endocarditis, and deletion of a manganese transporter results in loss of virulence (25). In addition, its NrdI and NrdF proteins belong to a group phylogenetically distinct from the E. coli, C. ammoniagenes, and B. subtilis class Ib enzymes, the only reported manganese-RNRs at the time we began this work.

Studies of the class Ib RNR have been hampered by the poor efficiency of MnIII$_2$Y$_2$ cluster assembly and consequently low catalytic activity of the enzyme. Our recent work on the B. subtilis class Ib RNR revealed that chromatographic removal of apo- and substoichiometrically metallated NrdF and identification and use of the endogenous reductant (Trx/TrxR) increased the activity of MnIII$_2$Y$_2$-NrdF to 1,475 units/mg, 18 times that measured for RNR isolated directly from B. subtilis. Furthermore, the activity of the MnIII$_2$Y$_2$-NrdF was 12 times that observed for FeIII$_2$Y$_2$ (125 units/mg) (47). In a similar study of the B. anthracis class Ib RNR, using the endogenous reductant Trx/DTT, the activity of the MnIII$_2$Y$_2$ was 10 times higher than the FeIII$_2$Y$_2$. However, the overall activity of MnIII$_2$Y$_2$-NrdF was only 65 units/mg, possibly associated with the inefficiency of manganese cluster assembly (6). With S. sanguinis RNR, the activity, 6,000 units/mg is very high, but the activity difference between the manganese- and iron-loaded NrdFs is only 5-fold using the endogenous reductant, NrdH/TrxR1/NADPH. This result is distinct from all other systems studied to date and raises an important question of which metal is used as a cofactor in vivo and how the organism’s environment might influence the enzyme’s metallation state.

Class Ib RNRS are found in actinobacteria, firmicutes, and α- and γ-proteobacteria, and recent studies have used bioinformatic analysis of the genomes of interest to identify candidate genes for the endogenous reductant(s) (6, 47). In some organisms, nrdH is found in an operon with nrdE and nrdF, whereas in the Bacillus genus, the nrdH is in a distant location (12). Recent studies of B. anthracis class Ib RNR identified several candidates for its endogenous reductant system as follows: two TrxS, three putative NrdHs, and three TrxRs. Biochemical analysis of the reductant’s efficiency $(k_{cat}/K_m)$ in recycling NrdE, accompanied by Western blot analysis of concentrations of the
**S. sanguinis Class Ib Ribonucleotide Reductase**

most interesting reductants, led to the conclusion that both Trx1 and NrdH could function in this capacity but that the former is the most likely candidate in vivo (6). Importantly, the reported V_{max} for the B. anthracis Mn^{III-2}Y is 45 to 65 units/mg was 100-fold lower than our S. sanguinis turnover number and independent of the reductants Trx1/DTT, Trx1/TrxR1, NrdH/DTT, and NrdH/TrxR1. Finally, Trx1 had no stimulatory effect at all on the turnover number of Fe^{III-2}Y, and therefore it was concluded that the manganese-loaded NrdF was the likely form of the class Ib RNR in vivo. Our studies of the B. subtilis class Ib RNR also support this hypothesis by showing that thioredoxins TrxA and YosR (NrdH-like) give a 10- and 5-fold stimulation of Mn^{III-2}Y activity, respectively, relative to DTT, but they stimulate Fe^{III-2}Y activity only 2-fold. In contrast, S. sanguinis NrdH significantly increased activity of both Mn^{III-2}Y and Fe^{III-2}Y 20-fold and 9-fold, respectively, relative to DTT.

Recently, we have measured an K_{m} of 25 nM for the B. subtilis class Ib LbE-NrdF (αβ-s) subunit interactions, which is 4-10-fold lower than the interactions in the E. coli class Ia RNR (K_{m} of 0.06 to 0.2 μM (62)). These results suggested that class Ib RNR can be assayed using a 1:1 ratio of subunits (47), instead of with an excess of one subunit over the other to ensure complete αβ complex formation (29). Our studies of S. sanguinis NrdF-NrdE, conducted using a 1:1 ratio of subunits in the concentration range from 0.001 to 0.1 μM (Fig. S8C), gave a K_{m} of 6.4 nM, similar to the B. subtilis class Ib LbR. The NrdE-NrdF interaction presents an opportunity to crystallize the active αβ complex, and this work is in progress.

The interaction of S. sanguinis NrdF with its NrdI is also of interest because they belong to a third phylogenetic subgroup that remained uncharacterized until our studies (24, 28, 51). This subclassification based on bioinformatics is supported by the measured K_{d} of 2.9 μM for S. sanguinis NrdI-NrdF interactions, distinct from tighter interactions measured for E. coli (<0.05 μM) and B. subtilis (0.6 μM) systems (16). Although our crystallization efforts have not yielded a structure of the S. sanguinis NrdF-NrdI complex, crystallization of the individual proteins has been successful. As suggested by sequence alignments (supplemental Fig. S2), the coordination environment of the S. sanguinis Mn^{III-2}-NrdF is much more similar to that of E. coli NrdF than the B. subtilis protein. The three distinguishing features between the E. coli and B. subtilis NrdFs are the H-bonding interactions between the Tyr residue to be oxidized in the active cofactor and the aspartate residue coordinated to Mn1, the presence of a H_{2}O molecule coordinated to Mn1, and the unusual bridging coordination of Glu^{158} to Mn1 and Mn2 (15, 53). Using all three criteria, S. sanguinis Mn^{III-2}-NrdF is similar to E. coli Mn^{III-2}-NrdF (Fig. 4). Another distinguishing feature between E. coli and B. subtilis NrdFs is the presence of a water-lined channel linking Mn2 to the FMN in NrdF containing a constriction created by Ser^{159} (Fig. 5A). The channel has been proposed to provide a pathway for the oxidant from the flavin in NrdF to the metal-binding site in NrdF. The crystal structure of S. sanguinis Mn^{III-2}-NrdF reveals the presence of a similar channel with a Thr^{158} counterpart to Ser^{159} at the constriction (Fig. 5B). The substitution for a bulkier side chain at this site may provide a more stringent selectivity filter for the oxidant. This property may be particularly critical in streptococci because these organisms accumulate high concentrations of H_{2}O_{2} (63, 64), and the observed constriction may restrict NrdF Mn^{III-2} site access by this molecule. In addition, the presence of this constriction might explain why Mn^{III-2}-NrdF cannot be activated directly with H_{2}O_{2} itself (4, 14).

The x-ray structure of S. sanguinis NrdI confirms predictions about the unique features of the streptococcal class Ib proteins. The longer sequence of S. sanguinis NrdI translates into an extended helix α1, an additional helix α1*, and an extended 70s loop when compared with structures of E. coli and B. subtilis NrdFs (Fig. 6A). These extensions could be involved in interaction with another protein, such as a NrdF reductase. A crystal structure of E. coli flavodoxin reductase (FlxR) (65) was used to construct a docking model with S. sanguinis NrdI (models generated with ClusPro (66)). As shown in Fig. 9A, a model was produced that places the long and bulky 70s loop and inserted helix α1* of NrdI in close contact with FlxR near its FAD cofactor. A similar model for interactions between FlxR and flavodoxin suggested that flavodoxin binds in a bowl-shaped pocket close to FAD with positively charged residues of FlxR positioned to interact with conserved negatively charged residues in the flavodoxin (65). A negative electrostatic surface potential near the FMN is a defining characteristic of flavodoxins, but NrdFs instead use positively charged residues to facilitate reduction of FMNH^{+} to FMN^{−} (67, 68). Based on analysis of the S. sanguinis NrdI electrostatic surface potential near its FMN cofactor, the negatively charged 70s loop does not seem to influence the positive electrostatic environment around the FMN and instead is localized at the outer surface of the protein (Fig. 7). Thus, we hypothesize that the negatively charged 70s loop may play a role in facilitating interaction between the positively charged pocket of FlxR (Fig. 9B) and the positively charged patch around FMN in NrdI.

Using the E. coli crystal structure of the NrdF-NrdI complex and sequence alignments (supplemental Fig. S2 and S3) with representative class Ib enzymes from the other subgroups, residues involved in forming the NrdF-NrdI interface were predicted for the B. subtilis and S. sanguinis systems. Interestingly, the NrdF portion of the protein-protein interface is more conserved than the NrdI portion. Therefore, differences in the K_{d} values measured for E. coli, B. subtilis, and S. sanguinis may be due to variations in the NrdI surface (supplemental Table S3) rather than differences in the NrdF component. Moreover, electrostatic analysis of S. sanguinis NrdF revealed that the
region surrounding the predicted NrdI-binding site is negative, which, in combination with the negatively charged 70s loop, could account for the particularly low affinity between these proteins.

An active cluster, Mn$^{III}$-2-Y-NrdF and Fe$^{III}$-2-Y-NrdF, can be assembled with 1 Y/β₂, and both forms exhibit relatively high activity with the physiological reductant NrdH/TrxR. However, the 3.5-fold difference in activities relative to the Mn$^{III}$-2-Y and Fe$^{III}$-2-Y observed with the B. anthracis and S. subtilis NrdFs, respectively, suggests that this organism might be able to stay active in vivo with either cofactor, with loading dependent on the growth environment. The accompanying paper by Rhodes et al. (30) demonstrates in a rabbit model for infective endocarditis that a strain of S. sanguinis in which nrdI has been deleted does not colonize heart valves, unlike the WT-strain. These studies thus provide the first evidence that a class Ib Nrd requires manganese under conditions in which the organism is pathogenic. Considering that streptococci and other pathogens, including enterococci, staphylococci, and Bacillus sp., contain class Ib RNR as their only aerobic RNR, prevention of Mn$^{III}$-2-Y$⁻$ formation in the class Ib RNR may be an attractive target for new antimicrobials.

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