Convergent allostery in ribonucleotide reductase

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Ribonucleotide reductases (RNRs) use a conserved radical-based mechanism to catalyze the conversion of ribonucleotides to deoxyribonucleotides. Within the RNR family, class Ib RNRs are notable for being largely restricted to bacteria, including many pathogens, and for lacking an evolutionarily mobile ATP-cone domain that allosterically controls overall activity. In this study, we report the emergence of a distinct and unexpected mechanism of activity regulation in the sole RNR of the model organism \textit{Bacillus subtilis}. Using a hypothesis-driven structural approach that combines the strengths of small-angle X-ray scattering (SAXS), crystallography, and cryo-electron microscopy (cryo-EM), we describe the reversible interconversion of six unique structures, including a flexible active tetramer and two inhibited helical filaments. These structures reveal the conformational gymnastics necessary for RNR activity and the molecular basis for its control via an evolutionarily convergent form of allostery.

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Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides, a conserved reaction that is fundamental to DNA-based life. As essential enzymes in central metabolism\textsuperscript{1,2}, RNRs have evolved two complex forms of allostery\textsuperscript{3,5}: specificity regulation, which is conserved in all RNRs, and activity regulation, which is canonically attributed only to RNRs with an evolutionarily mobile, regulatory domain known as the ATP-cone\textsuperscript{6}. In this study, we describe the unexpected emergence of a convergent form of activity regulation in the class Ib RNRs, a major subset of aerobic RNRs that lack ATP-cones\textsuperscript{7–11}. The evolution of class Ib RNRs is particularly relevant to medicine, as they are the primary aerobic RNRs used by a number of bacterial pathogens, such as *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*\textsuperscript{12}.

Nearly all aerobic organisms use class I RNRs, which consist of two subunits: \(\alpha\), which contains the catalytic site (Fig. 1a), and \(\beta\), which houses a subclass-dependent metal center (Fig. 1b). Enzyme activity requires the coordination of three processes: radical generation, nucleotide reduction, and re-reduction of the catalytic site. The first step involves reversible, long-range (~35 Å) radical transfer (RT) from a stable tyrosyl radical cofactor in \(\beta\) to a central cysteine in the catalytic site of \(\alpha\) (Supplementary Fig. 1a). The flexible C-terminus of \(\beta\) plays a critical role in this process by binding to \(\alpha\) and contributing a conserved tyrosine along the RT pathway\textsuperscript{13–16} (Supplementary Fig. 1c). Once the thiol radical is generated in \(\alpha\), nucleotide reduction proceeds via a conserved mechanism using two additional redox-active cysteines in the catalytic site as reducing equivalents\textsuperscript{2}. The resulting disulide is then reduced by a cysteine pair on the flexible C-terminus of \(\alpha\)\textsuperscript{17} (Supplementary Fig. 1d). Because RT is required for turnover, the active class I RNR complex is generally thought to adopt a compact conformation similar to a previously proposed symmetric \(\alpha_2\beta_2\) docking model\textsuperscript{18} (Supplementary Fig. 1a). However, the structural basis for RNR activity has not yet been elucidated at high resolution.

Allosteric regulation of RNRs plays a key role in maintaining the appropriate balance of intracellular nucleotides required for DNA replication fidelity. Class I RNRs reduce ribonucleoside diphosphates (NDPs, where \(N\) is any of the four bases, A, U, C, or G), with additional enzymatic steps converting the dNDP products into dNTPs. Substrate preference is allosterically coupled to the binding of a dNTP in the specificity or S-site (Fig. 1a), thus ensuring the balance of dNTP pools\textsuperscript{19}. In class I RNRs, the S-site is located at the interface of a canonical \(\alpha\)_2 dimer, hereafter denoted the “S-dimer” (Fig. 1a). Additionally, many RNRs from every class are able to regulate overall activity via a second allosteric site designated the activity or A-site (Fig. 1d). This site is housed in an evolutionarily mobile ~100-residue ATP-cone domain composed of a four-helix bundle and a three-stranded \(\beta\)-sheet cap, which is typically found at the N-terminus of the \(\alpha\) subunit\textsuperscript{8,11,20} (Fig. 1d, f). Binding of the substrate derivative ATP increases activity, while the downstream product, dATP, acts as a feedback inhibitor.

Most class I RNRs are classified into two subgroups on the basis of the radical-generating cofactor. Class Ia RNRs (NrdA = \(\alpha\), NrdB = \(\beta\)) are found in all domains of life and utilize a diferred tyrosyl cofactor. In contrast, class Ib RNRs (NrdE = \(\alpha\), NrdF = \(\beta\)) employ a dimanganic tyrosyl cofactor\textsuperscript{21–23} and are thought to have evolved from a Ia progenitor as a secondary aerobic RNR in bacteria\textsuperscript{24}. Studies of class Ia RNRs have shown that ATP-cone domains preferentially interface with other RNR chains (\(\alpha\) or \(\beta\)) upon binding of dATP to form ring-shaped oligomers that are proposed to inhibit activity by preventing long-range RT\textsuperscript{25–28}. Whereas most class Ia RNRs have at least one ATP-cone, class Ib RNRs are notable for sharing a characteristic truncated ATP-cone sequence at the N-terminus of \(\alpha\) (NrdE) and for lacking key A-site residues\textsuperscript{7,11,29} (Fig. 1e, f, orange). Consistent with this, class Ib RNRs have generally shown a lack of activity regulation\textsuperscript{7–10}.

Contrary to all other class Ib RNRs studied to-date, the sole RNR of *Bacillus subtilis* displays class Ia-like activity regulation\textsuperscript{30}.

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**Fig. 1** Allosteric sites of the *Bacillus subtilis* class Ib ribonucleotide reductase. **a** A 2.50 Å crystal structure of *B. subtilis* NrdE (\(\alpha\) subunit) obtained under activating conditions depicts an S-shaped dimer (“S-dimer”) interfacing at the “specificity” or S-site (lavender). A specificity effector TTP (green) is bound to the S-site, and activating nucleotides, ADP (pink) and ATP (salmon), are bound to two allosteric sites that evolved near the N-terminus of *B. subtilis* NrdE. A catalytically essential radical is generated at a central cysteine in the catalytic site, C382 (yellow sphere). **b** *B. subtilis* NrdF (\(\beta\) subunit) is dimeric and utilizes a dimanganic tyrosyl cofactor (purple spheres) to initiate radical chemistry (PDB: 4DR0)\textsuperscript{21}. A disordered region of the NrdF C-terminus (black dotted lines) is critical for radical transfer. **c** A recent structure of *B. subtilis* NrdE co-crystallized with dAMP (purple) depicts a partially inhibited, non-canonical “I-dimer” with the interface formed by the truncated ATP-cone (orange) (PDB: 6CGL)\textsuperscript{31}. In class Ia RNRs, ATP or dATP binds to the “activity” or A-site in the ATP-cone domain (orange). *B. subtilis* NrdE is unusual in that it displays activity regulation and binds dAMP (purple) in the N-terminally located I-site (PDB: 6CGL). **d** The partial N-terminal cone of class Ib RNRs (top) is structurally homologous to the last two helices of the canonical ATP-cone found in many class Ia RNRs (bottom) but lacks A-site residues.

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**ARTICLE**
Recently, we found that dATP inhibition in *B. subtilis* RNR is enhanced by the presence of a nucleotide with no previously documented role in RNR regulation: an endogenous deoxyadenosine monophosphate (dAMP) that co-purifies with NrdE\(^3\). A co-crystal structure revealed dAMP bound to an unexpected site in the truncated N-terminal cone domain to promote a dimerization at a non-canonical interface (Fig. 1c, PDB: 6CGL) rather than the expected S-dimer interface. We denote this dAMP-binding site the “I-site” (Fig. 1e) for its potential role in inhibition and correspondingly denote this structure the “I-dimer”. Although the structural basis for class I-like behavior was not elucidated, the new ligand and dimer implied a form of inhibition not yet seen in other RNRs\(^3\).

Motivated by this discovery, we applied a structural approach driven by small-angle X-ray scattering (SAXS) to elucidate the molecular basis for activity regulation in *B. subtilis* RNR. Because unique, thermodynamically stable states can be mathematically distinguished by SAXS\(^2\), we first used this technique to map the reversible and dynamic interconversion of six distinct oligomerization states in solution, including that of a flexible active complex and inhibited helical filaments (Supplementary Tables 1 and 2). Drawing on the conformational landscape established with SAXS, structures of individual states were systematically determined by cryo-electron microscopy (cryo-EM) and crystallography (Supplementary Tables 3–5). We find that *B. subtilis* RNR has arrived at a remarkably similar solution to the ATP-cone through the evolution of two unique allosteric sites that modulate catalytic activity by inducing contrasting quaternary interactions that either enable or prevent the full range of motions needed for RNR activity.

**Results**

**dATP binds to two allosteric sites to form a filament.** To examine the role of the unusual dAMP ligand in activity regulation, studies were conducted on both the dAMP-free and dAMP-bound species, hereafter referred to as “apo” and “holo” to describe the initial ligand state. Two types of SAXS experiments were conducted in this study (Supplementary Table 1). First, nucleotide and subunit titrations were performed to characterize structural transitions. Then, to obtain conformationally pure scattering profiles, SAXS was performed with in-line size-exclusion chromatography (SEC–SAXS), and the resultant datasets were mathematically decomposed with evolving factor analysis (EFA), a method we developed to separate dynamically exchanging states (Supplementary Table 2). We first confirmed with SEC–SAXS that in the absence of dATP, apo-NrdE is largely monomeric (Fig. 2a, blue), having a radius of gyration \(R_g\) of 29.5 ± 0.1 Å, whereas holo-NrdE is predominantly I-dimer, having an \(R_g\) of 44.0 ± 0.1 Å (Fig. 2a, orange). A systematic titration of dATP was then performed on both forms of NrdE at a physiologically relevant concentration of 4 \(\mu\)M with a saturating substrate concentration of 1 mM CDP. For both forms of NrdE, titration of dATP leads to a sharp increase in \(R_g\), approaching ~85 Å at inhibiting levels of dATP (50 \(\mu\)M). The \(R_g\) curve of apo-NrdE (Fig. 2b, gray) lags behind that of holo-NrdE (Fig. 2b, orange), suggesting that dAMP and dATP have partially overlapping effects. Singular value decomposition (SVD) yields two significant singular values for each individual dataset, whereas SVD of the two datasets combined yields three (Supplementary Fig. 2a). Because the monomer and I-dimer account for two singular values, the shared third singular value indicates that the final state is the same for both forms of NrdE.

To structurally characterize the dATP-induced oligomer, SEC–SAXS was performed on holo-NrdE with 100 \(\mu\)M dATP and 0.5 mM CDP in the running buffer (Supplementary Fig. 2b, top). The scattering curve of the predominant scattering component features a prominent secondary peak at \(q \sim 0.077\) Å\(^{-1}\) (Fig. 2a, red curve and star), suggestive of an extended oligomer with a hollow, cylindrical shape (Supplementary Fig. 2b, bottom). Together, these SAXS results indicate that the I-dimer is a component of a filament that forms in the presence of dATP. However, since the formation of this oligomer can occur without dAMP initially present, dATP is likely to be able to perform dAMP’s role, i.e. binding the I-site, in addition to binding another site.

**Cryo-EM structures of dATP-inhibited filaments.** Having obtained SAXS-based evidence for dATP-induced filament formation, structure determination was undertaken with cryo-EM (Supplementary Table 3, Supplementary Figs. 3–5). A 6 Å resolution cryo-EM map was obtained of holo-NrdE under inhibiting conditions (100 \(\mu\)M dATP, 1 mM CDP), revealing an unusual double-helix with a hollow center (Fig. 2d). In this structure, each helical strand is composed of alternating I-dimer and S-dimer interfaces, leading to a non-terminating chain of NrdE subunits (Fig. 2d). Importantly, the simulated scattering from this double-helical model captures the distinct secondary scattering peak observed in the experimental scattering (Fig. 2a, black dashed), indicating that this structure is a stable species in solution.

Because enzyme activity requires both \(\alpha\) and \(\beta\) subunits, cryo-EM was repeated with mixtures of holo-NrdE and NrdF (abbreviated as “NrdF”) under inhibiting conditions. The resultant cryo-EM map revealed a NrdF filament consisting of a single-helical NrdE strand (Fig. 2e, gray surface) with EM density corresponding to NrdF observed at higher contour levels filling the helical interior (Fig. 2e, green). The resolution of the reconstruction varied across the map, as high-resolution features were distinguishable in NrdE but not in NrdF (Fig. 2e, Supplementary Fig. 5b, d). Additional density was observed in a hydrophobic cleft of NrdE (Fig. 2e, f, green) that was previously shown to bind the \(\beta\) C-terminus in other class I RNRs. Using a previous structure\(^3\) as a guide, we were able to model the bound NrdF C-terminus as an eight-residue polyalanine chain (Fig. 2e, f, see the section “Methods”). In the NrdE-only filament, this cleft is unoccupied and partially buried by the double-helical interface formed by residues 660–665 (Supplementary Fig. 6a). Thus, the dissociation of the double-helical NrdF filament is likely a result of the NrdF C-terminus competing with this interface. These observations are consistent with a subunit titration performed with SAXS, in which addition of NrdF to the NrdE filament led to the immediate disappearance of the secondary peak (Fig. 2c, red star; Supplementary Fig. 4d).

For both filaments, excess density at the S-site was modeled as dATP (Fig. 2g). As dATP acts as a specificity effector for CDP reduction, its presence at this site explains the formation of the S-dimer interface between adjacent I-dimer units. Additionally, a distinct, protruding density is observed at the I-site that is best explained as a dATP that displaced dAMP (Fig. 2h). Side-chain densities in the I-dimer interface (Supplementary Fig. 6c) are modeled well by a previously reported crystal structure of the dAMP-bound I-dimer (PDB: 6CGL)\(^3\). A key feature of this interface is the “F47-loop” comprising residues 45–50, which immediately follows the partial ATP-cone sequence (Fig. 1f). The F47-loops from adjacent monomers interlock at the I-dimer interface, with F47 and H49 reaching across to interact with the opposing chain (Supplementary Fig. 6c). Our structures indicate that unlike dAMP, dATP can bind both allosteric sites, which further implies that any combination of a deoxyadenosine nucleotide at the I-site and a specificity effector at the S-site...
would induce dimerization at both interfaces and subsequent filament formation.

Density for the NrdF core is significantly weaker than the remainder of the NrdEF map (Supplementary Fig. 5b, d), but density for the NrdF C-terminus is observed at similar thresholds as NrdE (Fig. 2f). This discrepancy suggests that the NrdF core is flexibly linked to NrdE by NrdF C-termini, resulting in high occupancy but significant variability in orientation. The NrdF core volume is of the appropriate size and shape to be best explained by a NrdF dimer symmetrically centered with respect to a NrdE S-dimer, resulting in an $\alpha_2\beta_2$ asymmetric unit (ASU) (Fig. 2e, right). Attempts to refine the map from biased initial models with little gap between NrdE and NrdF invariably converged to have a pronounced gap of roughly 15–20 Å between the subunits (see the section "Methods"). Thus, instead of forming a buried interface with NrdE, the NrdF core density is on average placed far from the catalytic sites. Such a gap at the subunit interface would increase the distance between the buried filament.

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Activation by ATP entails the dissociation of the I-dimer. To investigate the structural basis for activation by ATP, we performed SAXS to examine the effect of ATP on the dAMP-induced I-dimer. Titration of ATP into 4 μM holo-NrdE led to a reduction in \( R_g \) indicative of I-dimer dissociation (Fig. 3a, orange circles). Likewise, SEC-SAXS of holo-NrdE with 1 mM ATP and 0.5 mM CDP yielded a single scattering component that is well described by the theoretical scattering of a monomer (Supplementary Fig. 7a). As a control, we confirmed that 4 μM apo-NrdE remains predominantly monomeric over the same range of ATP concentrations (Fig. 3a, gray squares). We thus find that at physiologically relevant concentrations, ATP disrupts the I-dimer interface.

We next examined the effect of ATP on the dATP-induced filament. Titration of ATP into 4 μM holo-NrdE pre-incubated with 50 μM dATP and 1 mM CDP led to a dramatic reduction in \( R_g \) (Fig. 3b, red circles), indicating that the extended filament dissociates in the presence of ATP. However, rather than dissociating into monomers, \( R_g \) converges to an intermediate value of \( \sim 36 \text{ Å} \), similar to the theoretical value for the S-dimer (39 Å). Apo-NrdE under comparably inhibited conditions converges to a similar \( R_g \) upon ATP addition (Fig. 3b, gray squares). These results suggest that although ATP and dATP competitively bind the I-site, dATP remains bound at the S-site.

To differentiate nucleotide binding at the S-site and I-site, we performed titrations with thymidine 5′-triphosphate (TTP), a specificity effector that is expected to exclusively bind the S-site (Supplementary Fig. 6b). Such conformational disorder of the individual NrdF cores while hindering access to the full range of motions needed for NrdF to act as a catalytic partner for NrdE. In particular, limited motion of NrdF can inhibit RNR activity if sequential steps of enzymatic turnover require the sampling of multiple binding modes between subunits.

**Fig. 3** SAXS reveals a complex interplay of four distinct oligomerization states of NrdE. In all plots, the theoretical \( R_g \) values of the I-dimer (46 Å, solid line), S-dimer (39 Å, dashed line), and monomer (27 Å, dotted line) are shown for comparison. a Titration of 0–1 mM ATP to 4 μM holo-NrdE (orange circles) leads to a reduction in \( R_g \) consistent with the dissociation of I-dimer to monomer and confirmed by SEC-SAXS (Supplementary Fig. 7a). In contrast, the \( R_g \) of 4 μM apo-NrdE (gray squares) remains constant up to 1 mM ATP. b Titration of 0–15 mM ATP to 4 μM holo-NrdE (red circles) or apo-NrdE (lavender squares) in the presence of 50 μM dATP leads to a decrease in \( R_g \) that converges to a value near the theoretical value of an S-dimer. c Titration of 0–500 μM TTP to 4 μM apo-NrdE (gray squares) leads to an increase in \( R_g \) that is suggestive of a monomer to S-dimer transition. A similar transition is observed with the addition of TTP to 4 μM holo-NrdE in the presence of 3 mM ATP (blue circles) and was further confirmed by SEC-SAXS (Supplementary Fig. 7b). d Titration of 0–500 μM TTP to 4 μM holo-NrdE in the absence of ATP leads to an increase in \( R_g \) and a final profile that resembles the dATP-inhibited filament ( Supplementary Fig. 2b, c). Source data are provided as a Source Data file.
site are both found in the N-terminus (orange) in close proximity to each other. Binding of ATP at the M-site induces F47 to its position in the I-dimer (gray, PDB: 6CGL)31.

Fig. 9a). R117 is thus important for discrimination of ribonucleotides and deoxyribonucleotides at this site.

Captured in the catalytic site of the 2.55 Å reduced dataset, revealing specificity of the I-dimer interface and that ATP formation of a large species, akin to the dATP-induced...

Crystallizing holo-NrdE with 5 mM Mg2+...resolution crystal structure of the S-dimer was obtained by co-crystallization of holo-NrdE with 1 mM ATP and 250 µM TTP showed that the entire elution can be decomposed as an interconversion of monomer and S-dimer with no contribution of the I-dimer (Supplementary Fig. 7b). In contrast, addition of TTP to 4 µM holo-NrdE in the absence of ATP induces the formation of a large species, akin to the ATP-induced filaments (Fig. 3d, Supplementary Fig. 2c). Combined, these results provide compelling evidence that specificity effectors, including ATP, favor the formation of the S-dimer interface and that ATP activates the enzyme by reversing the I-dimer-forming effects of dATP and dAMP.

Crystal structures reveal molecular basis for ATP recognition. With insight from SAXS (see the section “Methods”), a 2.50 Å resolution crystal structure of the S-dimer was obtained by co-crystallizing holo-NrdE with 5 mM Mg2+-ATP, 0.5 mM TTP, and 1 mM GDP. Electron density at the S-site could be unambiguously modeled as TTP coordinating a Mg2+ ion (Fig. 4a). Residues from both chains contribute to the S-site (Supplementary Fig. 8a), stabilizing the S-dimer.

Electron density at the I-site showed strong evidence for an ADP ligand displacing dAMP (Fig. 4b). As ADP was not present in the crystallization condition, we suspect that it is a hydrolysis product of ATP (see the section “Methods”). The bound ADP retains an H-bond to the α-phosphate and all of the adenine-specific interactions that were previously observed in dAMP-bound structures31 (Supplementary Fig. 8b, d). Although no interactions are observed with the β-phosphate, significant new interactions are observed involving the ribose. Specifically, R117 is within H-bonding distance of the 2′-OH of the ribose, N3 of the adenine ring, and E119 (Fig. 4c, gray residues; Supplementary Fig. 9b).

Interestingly, we also observed nearby electron density for an ATP ligand with the adenine ring positioned against the F47-loop of a second chain. Such a mechanism also explains the formation of a large species, akin to the dATP-induced crystallization condition, we suspect that it is a hydrolysis product of ATP (see the section “Methods”). The bound ADP retains an H-bond to the α-phosphate and all of the adenine-specific interactions that were previously observed in dAMP-bound structures31 (Supplementary Fig. 8b, d). Although no interactions are observed with the β-phosphate, significant new interactions are observed involving the ribose. Specifically, R117 is within H-bonding distance of the 2′-OH of the ribose, N3 of the adenine ring, and E119 (Fig. 4c, gray residues; Supplementary Fig. 9b).

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Interestingly, we also observed nearby electron density for an ATP ligand with the adenine ring positioned against the F47-loop of a second chain. Such a mechanism also explains the formation of a large species, akin to the dATP-induced crystallization condition, we suspect that it is a hydrolysis product of ATP (see the section “Methods”). The bound ADP retains an H-bond to the α-phosphate and all of the adenine-specific interactions that were previously observed in dAMP-bound structures31 (Supplementary Fig. 8b, d). Although no interactions are observed with the β-phosphate, significant new interactions are observed involving the ribose. Specifically, R117 is within H-bonding distance of the 2′-OH of the ribose, N3 of the adenine ring, and E119 (Fig. 4c, gray residues; Supplementary Fig. 9b).
preferred ligand. In support of this, we screened 27 diffraction-quality crystals grown with various combinations of ATP, TTP, GDP, and CDP, and only those grown with GDP displayed electron density at the M-site. For example, no density was observed at this site in a 2.95 Å structure of the S-dimer obtained with CDP instead of GDP in the crystallization condition (Supplementary Fig. 9c, d). The non-hydrated nature of the ATP bound to this site further suggests that it was introduced when the crystal was soaked in freshly made cryoprotectant solution containing 5 mM ATP and that it may have displaced a GDP that primed this site for nucleotide-binding. Regardless, an important consequence of either ATP or GDP bound to the F47-loop is that the F47 side-chain flips inward by ~90° to form a stacking interaction with the purine ring (Fig. 4d). With F47 in this conformation, it is unable to participate in the formation of an I-dimer interface. We thus define this allosteric site as the “M-site” for its proposed ability to cause dissociation of I-dimers into monomers (Figs. 1a, 4d).

Together, our crystal structures and SAXS results suggest that inhibition by I-dimer formation requires two conditions to be met: the I-site must be loaded with a deoxyadenosine nucleotide, and the M-site must be empty in both chains. Consistent with this, our cryo-EM maps of the dATP-induced filament display empty pockets at the M-site (Supplementary Fig. 6c, purple stars). However, the absence of nucleotide in the M-site of our 2.95 Å S-dimer structure suggests that ATP’s activating effect is primarily exerted by displacement of deoxyribonucleotides at the I-site.

Visualization of the α C-terminus in the active site. In all class I RNRs, the α C-terminus is thought to enter the catalytic site after each turnover to allow for the tail cysteines to regenerate the reducing cysteines via thiol-disulfide exchange17 (Supplementary Fig. 1d). Unexpectedly, we were able to trap the final six residues of the NrdE C-terminus in the catalytic site of our 2.50 Å structure due to partial disulfide formation between the tail cysteines (C695, C698) and two catalytic-site cysteines (C382, C170) (Supplementary Figs. 10a, 11). These linkages, particularly that involving C695 and the initial site of the catalytically essential thyl radical, C382, are likely artifacts of sample oxidation. However, they allowed us to capture the flexible C-terminus, which has never before been visualized in any class I RNR.

Furthermore, we were able to use X-ray reduction of the same crystal to determine a 2.55 Å resolution structure depicting the reduced catalytic site (Fig. 4g, Supplementary Table 4, Supplementary Fig. 11). With the exception of the terminal residue V700, for which the side-chain does not participate in any polar interactions, all of the residues involved in binding the disulfide-trapped C-terminus are highly conserved in NrdE sequences (see the section “Methods”) and nearly superimposable with corresponding residues in previous structures of NrdE with no C-terminus bound29,31.

ReDUCTION of the catalytic-site cysteines is expected to require two sequential S2→2 nucleophilic substitutions. Assignment of a Salmonella typhimurium NrdE structure29 (Supplementary Fig. 10c, dark gray) with the C-terminus from our X-ray-reduced structure (Supplementary Fig. 10c, purple sticks) places C698 within 3 Å of the oxidized C178–C415 pair in the S. typhimurium catalytic site (equivalent to C170–C409 in B. subtilis numbering). These three cysteines form a triad with optimal geometry for disulfide exchange, with C698 well positioned to attack C170. Thus, the tail-binding interactions involving C698, C170, and C409 appear to be physiologically relevant for the first step of re-reduction, in turn likely resulting in the oxidized C698–C170 we observe in our disulfide-trapped structure. In contrast, C695 is too far from C698 to perform the second step of re-reduction (Supplementary Fig. 10d) and is instead positioned closer to C382. A possible explanation is apparent in our X-ray-reduced structure (Fig. 4g), in which we resolve an additional interaction: an H-bond between the residue adjacent to C695 (S694) and a non-conserved residue near the catalytic site (S246). Under physiological conditions, we expect C695 to move closer to C698, pulling the remainder of the C-terminus further into the catalytic site.

Our structures also provide insight into the relationship between re-reduction and RT. In the current model for thyl radical generation in class I RNRs, two tyrosines stack over C382 to form a π–π dyad required for co-linear proton-coupled electron transfer (PCET) (Supplementary Fig. 1c)38,39,40. These tyrosines (Y683/Y684) are located in the β strand preceding the C-terminal tail sequence of NrdE. In structures of the class Ib RNR from S. typhimurium29,35, this strand is observed forming a β-sheet with the adjacent β strand (Supplementary Fig. 12a, yellow), whereas in ours; it is partially unzipped from the β-sheet, allowing the C-terminus to reach the catalytic site (Supplementary Fig. 12b, yellow). The Y684 side-chain flips out by ~180° to accommodate the resulting bend. These observations suggest that binding of the NrdE C-terminus is associated with the Y–Y dyad becoming unstacked, and hence, re-reduction of the catalytic site and RT cannot occur concurrently. Such a conformational gating mechanism would allow RNR to coordinate the multiple processes required for activity.

Active NrdE is a flexible tetramer. To gain insight into the conformational ensemble of the active complex, NrdE was examined with SEC–SAXS under various non-inhibiting conditions using a C382S mutant of NrdE to prevent changes in nucleotide concentrations (see the section “Methods”). We initially found that without both ATP and a specificity effector present, stoichiometric combinations of holo-NrdE and NrdF resulted in complex mixtures of oligomers that often include species that are larger than an αβ2 tetramer. Under S-dimer promoting conditions (1 mM ATP, 250 µM TTP), however, the scattering was dominated by a single species with a molecular weight estimate of 246 kDa32, consistent with αβ2 (Fig. 5a, blue curve; Supplementary Fig. 13).

To interpret the scattering, we first considered the widely accepted symmetric αβ2 docking model18 (Supplementary Fig. 1a) as a starting model. Using the program AllosMod-FoXe40, missing residues were modeled, and simulated conformers consistent with the starting model were sampled to minimize the fit to the experimental scattering. A poor fit was obtained (Fig. 5a, purple dashed; χ2 = 20.43), indicating that the solution ensemble cannot be captured by the local energy landscape of the docking model (Fig. 5b, bottom). A similarly poor result was obtained from an “expanded” αβ2 starting model with a pronounced gap at the subunit interface generated by fitting a NrdF dimer crystal structure (PDB: 4DR0)21 into the αβ2, ASU of the NrdE cryo-EM map (Fig. 2e, right and Fig. 5a, orange dashed; χ2 = 18.55). We thus considered an alternative αβ2 conformation depicted by a 4.0 Å resolution crystal structure of the class Ib RNR from S. typhimurium53 in which β2 is angled asymmetrically with respect to the α S-dimer, exposing the catalytic sites (Supplementary Fig. 1b). Remarkably, this starting model resulted in the best fit (Fig. 5a, black dashed; χ2 = 2.25). The final model remains asymmetric overall, but the angle between the two subunits is reduced by 14° (Fig. 5b, top). Thus, although B. subtilis RNR must sample a compact conformation for long-range RT, our analysis suggests that an asymmetric arrangement is a significant component of the conformational ensemble in solution.
Discussion

Based on our SAXS, EM, and crystallography results, we propose an allosteric model for B. subtilis RNR involving six distinct NrdE-containing species (Fig. 6). At physiologically relevant protein concentrations (low μM), nucleotide-free NrdE is primarily monomeric. The I-site is specific to adenine nucleotides where binding of deoxyribonucleotides (dAMP and dATP in particular) leads to a partially inactive I-dimer. In contrast, binding of any specificity effector (dATP, dGTP, or TTP) to the S-site stabilizes the S-dimer interface. Thus, the combination of these nucleotides leads to a NrdE filament having alternating dimer interfaces, with the inhibitor dATP uniquely able to bind both sites. Although NrdF is able to associate with the NrdE filament through its C-termini, when confined within the helical interior, we propose that it is unable to access the sequential motions necessary for interaction with NrdE as a catalytic partner. Conversely, the primary activating effect of ATP is to destabilize the I-dimer interface by displacing deoxyribonucleotides at the I-site. As ATP has a low affinity for the S-site, the combination of a specificity effector at the S-site and ATP at the I-site favors S-dimer formation. Likewise, ATP is able to dissociate the NrdE filament at the I-dimer interfaces, producing αβ₂ complexes as the active form. In this oligomerization state, NrdF is able to access the full range of motions needed for RNR activity.

Together with prior work31, our results indicate that two unique allosteric sites have evolved in B. subtilis RNR. In particular, the I-site, which is formed by residues in the α₂-helix of the truncated ATP-cone and adjacent secondary structural elements, has evolved adenine specificity and sugar discrimination (Fig. 4, Supplementary Fig. 8). However, it shows no apparent discrimination for the number of phosphates, perhaps indicating that unlike the S-site, it has yet to evolve specificity for triphosphates, the final products of deoxyribonucleotide metabolism. Alternatively, the ability of the I-site (but not S-site) to tightly bind dAMP in B. subtilis RNR31 may be advantageous as an additional tuning dial for activity regulation. Although not shown in our scheme (Fig. 6), our results also suggest a secondary mechanism for activation involving the M-site. Because the M-site is coupled to the F47-loop, ATP and possibly GDP binding here would directly interfere with I-dimer formation. Notably, it has been shown by analytical ultracentrifugation that in the absence of GDP, B. subtilis NrdE is monomeric12. However, as apo-NrdE is preferentially a monomer, binding of nucleotide at the M-site does not appear to be a requirement for I-dimer dissociation.

Our crystal structures provide key insight into the requirements of an active complex. The α-C-terminal residues that we observe (695-CLSCVV-700) occupy the catalytic site in a conformation that is remarkably similar to the binding mode of nucleotide substrates observed in other class I RNRs, with the terminal carboxylate mirroring the interactions of a substrate β-phosphate (Supplementary Fig. 10a, b)19. Since catalytic-site reduction by the α C-term is likely a derived trait of class I and II RNRs81, this similarity suggests that the C-terminus evolved to mimic substrate binding. These structures also imply that when the α C-terminus is bound, RT cannot occur due to the coupled unstacking of the Y-Y dyad. Conversely, alignment of our structures with the S. typhimurium αβ₂ structure33 suggests that when the Y-Y dyad is stacked, the associated β-sheet (Supplementary Fig. 12b, yellow cartoon) presents a grooved surface that may serve as a potential binding site for the β C-terminus. Such a binding mode would bring Y307 on the flexible region of the β C-terminus in close proximity to the Y-Y dyad in an arrangement thought to be necessary for RT (Supplementary Fig. 1c). Together, these structures imply that binding of the α and β C-termini to the catalytic site may be mutually exclusive and that significant structural dynamics are required for RNR activity.

Consistent with this notion, our SAXS results provide evidence for an αβ₂ conformation that is asymmetric like the S.
have recently been reported, the dATP-inhibited interface. Finally, addition of NrdF to the S-dimer leads to formation of an inhibited double-helical NrdE. city effectors such as dATP can displace dATP from the I-site and induce dissociation of the I-dimer. When positional con-formation of an inhibited double-helical NrdE I-dimer formation that is important for activity. Additionally, the NrdEF structure. In this structure, one lobe of NrdE with this, the residues involved in the I-site and F47-loop are fully conserved. ATP-cone has been reported to display such behavior. Consistent with this, the residues involved in the I-site and F47-loop are fully conserved in only a small subset of class Ib RNRs (see the section “Methods”). We thus hypothesize that truncation of the NrdE structure. In this structure, one lobe of NrdE with this, the residues involved in the I-site and F47-loop are fully conserved. ATP-cone has been reported to display such behavior. Consistent with this, the residues involved in the I-site and F47-loop are fully conserved in only a small subset of class Ib RNRs (see the section “Methods”). We thus hypothesize that truncation of the ATP-cone in the class 1a progenitor of class Ib RNRs rendered the domain vestigial and that the original class Ib RNR did not need activity regulation because it was the organism’s secondary RNR and was only expressed under iron-poor conditions. Because the class Ib enzyme of B. subtilis is the sole RNR used by the organism, evolution of new binding sites and allosteric mechanisms within the truncated ATP-cone domain may have been advantageous. However, as class Ib RNRs are used by a number of pathogens, it is also worth noting that loss of RNR allostery, which has been linked to increased mutation rates, may also provide a selective advantage in evolving therapeutic resistance.

Perhaps the most striking result of this study is that with B. subtilis RNR, nature has arrived at a remarkably similar solution to the ATP-cone, whereby binding of dATP induces a new oligomeric interface that disrupts the protein–protein interactions needed for RT between α and β. The convergence of these two evolutionary routes with disparate origins reveals the importance of nucleotide homeostasis as a selective pressure, as well as the flexibility by which new protein–protein interactions can evolve.

Methods

Expression and purification of B. subtilis RNR proteins. Protein expression and purification were performed following established protocols. Tagless NrdE (both wild type and C382S variants) was produced using SUMO fusion technology with pE-SUMO-nrdE vectors developed in a previous study. His-Fmt-Smt3-tagged NrdE was overproduced in BL21 (DE3) cells and purified with a nickel or cobalt affinity column followed by anion exchange chromatography on a Q-Sepharose column. The His-Fmt-Smt3 (SUMO) tag was cleaved using SUMO protease and separated from the tagless NrdE protein with a second nickel affinity chromatography step. To separate nucleotide-free (apo-NrdE) and dAMP-bound (holo-NrdE) fractions, a final high-resolution anion exchange chromatography step was conducted using a MonoQ 10/100 Gl column (8 mL). His-tagged apo-NrdE was produced in a similar manner but without the tag cleavage steps and with the addition of 100 µM 1,10-phenanthroline in the cell lysis buffer to prevent mis-metallation. Apo-NrdE was then reconstituted with Fe(III) or Mn(III) according to previously established protocols. Briefly, iron reconstitution was performed by anaerobic addition of Na2Fe(SO4)2 to apo-NrdE followed by incubation with oxygen-saturated buffer. For manganese reconstitution, MoCl4 is instead used, concomitant with the hydroquinone form of the accessory protein Nrdl, to generate the active Mn cofactor. In both cases, holo-NrdE is purified from apo- and mismetallated NrdE by a subsequent anion exchange chromatography step using a MonoQ column. All protein concentrations are given as monomer concentrations. Unless otherwise noted, all studies were done in the standard assay buffer: 50 mM HEPES pH 7.6, 150 mM NaCl, 15 mM MgCl2, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 1% or 5% (w/v) glycerol.

Sequence conservation. A multiple sequence alignment of NrdE sequences from the RNRs was performed in Muscle and analyzed in Maltan (The Math-works). Of the 5216 non-redundant sequences, 151 (3%) show full conservation of residues in the NrdE (Tables 1 and 2). Small-angle X-ray scattering (SAXS/WAXS) images were simultaneously collected on two Pilatus 100K detectors covering a range of q = 0.01–0.7 Å−1. Here, the momentum transfer variable is defined as q = 4π/λ sin θ/2, where λ is the X-ray wavelength and θ is the scattering angle. Data processing at the beamline was performed in BioXTAS RAW. Final data processing and analysis were performed using ATSAS and in Maltan following established protocols. Briefly, scattering images were integrated about the beam center and normalized by transmitted intensities measured on a photodiode beamstop. The integrated protein scattering profile, I(q), was produced by subtraction of background buffer scattering from the protein solution scattering. Radii of gyration (Rg) were estimated with Guinier analysis, and pair distance distribution analysis was performed in GNOM. Error bars associated with Rg values are curve-fitting uncertainties from Guinier analysis. Molecular weight estimation was performed using a Porod invariant method implemented in SANSMoW. Small-angle X-ray scattering experiments, proteins were first studied at multiple concentrations to examine inter-particle effects and oligomerization. Apo-NrdE (both wild-type and C382S) remained monomeric over a wide range of
concentrations (1–20 μM). Holo-NrdE displayed concentration-dependent oligomerization, with C382S having a greater tendency to dissociate than wild-type, likely due to its inability to form Fe-NrdF. detergent conditions, both constructs behaved identically. A near-physiological protein concentration of 4 μM was chosen for subsequent titration to minimize concentration effects while maintaining a reasonable signal-to-noise ratio. Where required, the subunit titration was performed with Fe-NrdF, which is structurally interchangeable with Mn-NrdF and can be produced with greater yield. For all titration experiments, background subtraction was performed with carefully matched buffer solutions containing identical concentrations of nucleotides following established protocols. For each measurement, 40 μL of sample were prepared and centrifuged at 14,000 g at 4°C for 10 min immediately before loading into an in-vacuum flow cell kept at 4°C. For each protein and buffer solution, 20 ± 2 exposures were taken with sample oscillation to limit radiation damage then averaged together to improve signal. Data was averaged in [software].

E. coli R-705 were constructed by aligning a structure of the NrdE S-dimer determined in this work. Titration experiments, background subtraction was performed with carefully matched buffer solutions containing identical concentrations of nucleotides following established protocols. For each measurement, 40 μL of sample were prepared and centrifuged at 14,000 g at 10 min immediately before loading into an in-vacuum flow cell kept at 4°C. For each protein and buffer solution, 20 ± 2 exposures were taken with sample oscillation to limit radiation damage then averaged together to improve signal. Data was averaged in [software].

Swapping of static structures consistent with the starting model was performed with a GE Superdex 200 Increase 3.2/300 (2.4 mL), Superdex 200 5/150 GL (3 mL), or Superdex 200 Increase 10/300 GL (24 mL) column operated by a GE Akta Purifier at 4°C with the elution flowing directly into an in-vacuum X-ray sample cell (Supplementary Tables 1 and 2). To account for a ~10-fold dilution of the sample during elution, 50–75 μL samples were prepared with 40–80 μM protein in assay buffer with nucleotides. Samples were then centrifuged at 14,000 g for 10 min at 4°C before loading onto a column pre-equilibrated in a matched buffer. Samples were eluted at flow rates of 0.05–0.1 mL min⁻¹ for the 3.2/300 column and 0.15–0.2 mL min⁻¹ for the 5/150 and 10/300 columns. Ten 2-s exposures were collected throughout elution until the elution profile had returned to buffer baseline, and scattering profiles of the elution buffer were averaged to produce a background-subtracted SEC–SAXS dataset. Data were analyzed by the [CTF program] and [software] using custom [software] code.[25] [PDI] allows for mathematical separation of partial and bulk scattering. The phase agreement parameter, the exchange agreement parameter, and the overall correlation parameter all iterate to converge if the density map is a single density map. The density map of the NrdE filament was reconstructed using the helical symmetry parameters: the inter-helix symmetry (74.24 Å, 0.1 mL min⁻¹) and rotational degree-of-freedom was included in the NrdE filament refinement. According to the [CTF program] algorithm, each “seed” raw image receives its initial alignment from the corresponding class average for the subsequent processing. A phase-residual-based projection-matching algorithm was used in the particle alignment. In each refinement cycle, the alignment parameters (X/Y/Z-translations and three Euler angles) of each particle frame were adjusted to minimize the local cross-correlation of 50 iterations, the 3D reconstruction converged to 4.8 Å resolution, as determined by the standard 0.143 FSC criterion. However, based on features of our map as well as our map-model FSC, we believe the NrdE map to actually be closer to 6 Å and thus report it as such. The double-helix NrdE filament has two sets of helical symmetry parameters: the inter-helix symmetry (74.24 Å, −81.28°, where the negative sign indicates a flip in handedness) and intra-helix symmetry (37.12 Å, 139.36°).

**Electron microscopy of the NrdE filament.** Cryo-EM samples of the NrdE filament were prepared by mixing 20 μM holo-NrdE with 20 or 40 μM Mn-reconstituted NrdF in assay buffer with 100 μM dATP and 1 mM CDP, prior to four-fold dilution with nucleotide-containing buffer. 3.5 μL of the diluted sample (2 μL for 5 or 10 μM NrdE samples and 2 μL for 200 mesh C-flat grids (Protocols, 2 μm hole size), blotted with Whatman #1 filter paper (GE Healthcare), and plunged-frozen in liquid ethane using an EM-500 (Leica) at 95% relative humidity. A subset of the grids was pre-coated with a support film of continuous, amorphous carbon by flotation of cleaved mica. For these grids, the same grid protocol was followed but the carbon film was instead coated on a 200 κV Talos Arctica cryo-electron microscope (FEG). Particles from 2843 images were used in the final reconstruction, of which 446 images were obtained over continuous carbon film. Images were recorded using a K2 Summit camera (Gatan) operating in counting mode: nominal 1.05 Å per pixel, five frames per second, with flux ranging from 5 to 12 e⁻ per pixel per second. 95% of images used had defocus between 0.8 and 2.5 μm. A representative image is shown in Supplementary Fig. 4a. Movie frames were aligned with MotionCor2.[25] CTF parameters were refined with Gctf.[25] Filaments were manually annotated and extracted using the helix boxer in RELION.[25] The annotator selected regions, where filaments, aggregates, or contaminants. For extraction, the initial parameters quickly converged to ~75 Å unit rise and 80° unit rotation (Supplementary Fig. 3c). Because the long dimension of a NrdE dimer is ~75 Å, according to the previously determined crystal structure (PDB: 6CGL), we predicted that this repeating pattern would contain nine dimeric units over a span of 675 Å. We thus estimated the unit rotation angle to be around θ = 360°/N = 40°, where N = 1, 2, 3, ... is the number of helical turns. A test of helical reconstruction based on these parameters converged to ~75 Å unit rise and the unit rotation (where N = 2), which served as the initial helical parameter in the subsequent reconstruction and refinement.

The first step of refinement employed DPC class averages as the “particle stack” input. Taking the above initial model as reference, a projection-matching algorithm was used to align all DPC class averages and also used in the subsequent 3D refinement. For each particle frame, the crystallographic resolution, divided into two sets of helical symmetry parameters: the inter-helix symmetry (74.24 Å, −81.28°, where the negative sign indicates a flip in handedness) and intra-helix symmetry (37.12 Å, 139.36°). The density map of the NrdE filament was reconstructed using the helical parameter: (Supplementary Fig. 3c). To improve the resolution of the helical reconstruction, the helical parameters were re-evaluated at the end of each iteration of refinement using the "helical symmetry solver" function in RELION, in which a local search (within 2 Å and 2°) around the current helical parameters was performed to identify the maximum density cross-correlation within a cylindrical mask over two repeating units (150 Å) in the middle of the double-helix density map (Supplementary Fig. 3d). The resulting helical parameters were further refined with the DPC algorithm, which served as the initial helical parameter in the subsequent reconstruction and refinement.

After 50 iterations, the 3D reconstruction converged to ~5.8 Å resolution (Supplementary Fig. 3c). To improve the resolution of the helical reconstruction, the helical parameters were re-evaluated at the end of each iteration of refinement using the "helical symmetry solver" function in RELION, in which a local search (within 2 Å and 2°) around the current helical parameters was performed to identify the maximum density cross-correlation within a cylindrical mask over two repeating units (150 Å) in the middle of the double-helix density map (Supplementary Fig. 3d). The resulting helical parameters were further refined with the DPC algorithm, which served as the initial helical parameter in the subsequent reconstruction and refinement.

After 50 iterations, the 3D reconstruction converged to 4.8 Å resolution as determined by the standard 0.143 FSC criterion. However, based on features of our map as well as our map-model FSC, we believe the NrdE map to actually be closer to 6 Å and thus report it as such. The double-helix NrdE filament has two sets of helical symmetry parameters: the inter-helix symmetry (74.24 Å, −81.28°, where the negative sign indicates a flip in handedness) and intra-helix symmetry (37.12 Å, 139.36°).
internal structure when 2D classification was used to select a subset of particles for analysis, but the resolution was 0.07 Å worse. It is possible that data sorting was superfluous because manual boxing of helices pre-selects for high-quality particles. Because cryoSPARC v2 does not support helical symmetry, we used a large box size (480 pixels) containing many NrdEF repeats for processing as an asymmetric single particle. Helical symmetry emerged naturally in the C1-referenced map. We then cropped the final reconstruction to display the central NrdEF repeats to avoid radial and/or edge effects in the box boundary. The handedness of the final map was flipped based on the handedness of published NrdE structures.

To test for consistency, the full dataset was also processed in cryoSPARC v1.67 and in RELION30, with a box size of 256 pixels and considering helical symmetry in the case of RELION. At the resolution of the CryoSPARC output maps, we did not observe notable differences in NrdE internal structure from package to package. We also simulated several biased models using UCSF Chimera31. These include a NrdE-only map made by erasing NrdE density using SEGGER33, a filament based on an asymmetric S. typhimurium NrdE crystal structure (PDB: 2BQI)35 (Supplementary Fig. 1b) and simulating electron density with molmap; and a filament based on a symmetric docking model36 (Supplementary Fig. 1a), also simulated with molmap. 3D autorefinement was performed in RELION using these simulated maps as references to test whether a biased reference would cause refinement to converge to a different local minimum. In all cases, refinement converged on a map with a pronounced gap at the substrate interface. The maps resulting from biased models were not carried forward to any other refinement, and no initial models were used in creation of the deposited NrdE map.

The final helical parameters were consistent from package to package. The helical twist measured at 88.6° (cryoSPARC v2) or 90.9° (RELION), and helical rise was measured at 73.8 Å (cryoSPARC v2) or 73.2 Å (RELION).

Electron microscopy model refinement. Model refinement was first performed with the higher-resolution map of the dATP and NrdEF filament. A helical model for the NrdE monomer was built using the crystal structure of the dAMP-bound 1-dimer (PDB: 6CGL)37 as the basis. Residues involved in forming the S-dimer were re-modelled based on a symmetric docking model (Supplementary Fig. 1a), also simulated with molmap. 3D autorefinement was performed in RELION using these simulated maps as references to test whether a biased reference would cause refinement to converge to a different local minimum. In all cases, refinement converged on a map with a pronounced gap at the substrate interface. The maps resulting from biased models were not carried forward to any other refinement, and no initial models were used in creation of the deposited NrdE map.

The final helical parameters were consistent from package to package. The helical twist measured at 88.6° (cryoSPARC v2) or 90.9° (RELION), and helical rise was measured at 73.8 Å (cryoSPARC v2) or 73.2 Å (RELION).

SAXS results had suggested that ATP is a poor specificity effector, we speculated that we could stabilize this packing arrangement by addition of TTP to the pre-mature crystals. To this end, we carried out NrdE single crystal analysis using the same crystal form (P212121;2);2) were obtained in 4–5 days when 4.5 mg mL−1 holo-NrdE was co-crystallized with 5 mM Mg2+–ATP, 0.5 mM TTP, and 5 mM CDP. Use of the preferred substrate for TTP (1 mM GDP) in place of CDP led to the appearance of large crystals in 2 days. These crystals have the same packing arrangement, but with a different handedness of packing in the ASU, which forms a S-dimer with a symmetry molecule. Thus, systematic changes to the nucleotide mixtures led to progressive improvement in crystalline order and, in turn, resolution. Crystals for data collection were grown by vapor diffusion from 5.4 mg mL−1 holo-NrdE in 50 mM HEPES pH 7.6, 50 mM NaCl, 5 mM MgCl2, 2 mM TCEP, and 1% glycerol, supplemented with 5 mM ATP, 500 µM TTP, and either 1 mM GDP or 5 mM CDP. The protein solution was incubated for 10 min with freshly added nucleotides prior to being mixed in a 1:1 hanging drop with a precipitating solution of 6% PEG 3350, 1% w/v tryptone, and 50 mM HEPES at pH 6.9 or 7 for crystals grown in the presence of GDP and CDP, respectively. Crystals were cryoprotected by soaking for 5–10 s in well solution with 8% v/v sucrose, 2% w/v glycerol, 8% v/v ethylene glycol and supplemented with nucleotides, TCEP, and MgCl2 adjusted to the same concentrations used in the original protein solutions.

Data collection was performed at CHESS beamline F1 on a Pilatus 6M detector at 100 K, a wavelength of 0.9775 Å, and with 0.2° oscillation step. Diffraction images were integrated using either XDS76 (P4_21_2), 2.1.16 or iMosflm77 (P21_21_2), and were scaled and merged using AimLESS78. Phases were estimated by molecular replacement in Phaser79 using the apo-NrdE monomer structure (PDB: 6CGG)30 as the search model. Model building in Coot80 was performed iteratively with positional and B-factor refinement in Phenix81. Model building was performed with the Molprobity server and the wwPDB validation server. Data collection and model refinement statistics are shown in Supplementary Table 4. Figures were generated using PyMol (Schrödinger, LLC). Polder omit maps were generated using Phenix82. Ligand interaction diagrams were generated using LigPLOT++. The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 6MT9, 6MVE, and 6MV9.

To produce the crystal structures of disulfide-trapped (PDB: 6MT9) and X-ray-reduced (PDB: 6MVE) NrdE S-dimer, a highly redundant dataset was collected on a single crystal grown from the TTP/ATP/GDP condition to yield two structures: a 2.50 Å disulfide-trapped structure and a 2.55 Å X-ray-reduced structure from the second half of data collection (Supplementary Table 4). The 2.50 Å structure was refined in a similar manner as above. Clear electron density was observed at the M-site, the I-site, and catalytic site. The M-site was modeled as a peptide based on its shape and density at the catalytic site. As in the 2.95 Å structure, the electron density at the I-site was more consistent with ADP than with ATP. At the M-site, both ATP and GDP were initially considered. While the electron density is less resolved here than at the I- and catalytic sites, there is a notable lack of density for the N2 amine of a GDP, and there is also density for a γ-phosphate. Thus, ATP was modeled into the density rather than GDP. We note, however, that the binding site is likely to be able to bind GDP as well, and that the ATP may have displaced GDP when soaked in with cryoprotectant.

Density at the catalytic site was modeled as a peptide based on its shape and connectivity to catalytic-site residues C382 and C170. The C-term was then restrained via iterative manual restraint refinement in Coot and refinement in Phenix, using the disulfide bridging residues C695 and C698 as anchor points. Interestingly, these disulfides were not reduced by fresh TCEP that was introduced in the cryoprotectant. However, we were able to obtain a largely reduced structure by collecting a redundant diffraction dataset. Using these later frames, the structure was refined using rigid body refinement from the 2.90 Å oxidized structure as a starting model. The resulting 2.55 Å dataset (Supplementary Table 4) is at slightly lower resolution while retaining good merging statistics. The structure is virtually unchanged except that cysteines 382, 170, 695, and 698 were better modeled in the reduced form. However, we note that there is evidence for occupancy in both the 2.50 and 2.55 Å structures (Supplementary Fig. 11). The final models for the 2.50 Å disulfide-trapped and 2.55 Å X-ray-reduced NrdE structures include residues 6–238, 246–685, and 695–700 (2.50 Å) or 694–700 (2.55 Å); 1 molecule of TTP; 1 molecule of ADP; 1 molecule of ATP; 1 Mg2+; and 194 (2.50 Å) or 133 (2.55 Å) water molecules.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability

Coordinates and structure factors for crystal structures have been deposited in the Protein Data Bank under the following accession codes: videi-trapped S-dimer (PDB 6MT9), X-ray-reduced S-dimer (PDB 6MVE), and S-dimer with empty M-sites (PDB 6MV9). EM structures and associated atomic models have been deposited in the Electron Microscopy Data Bank and the Protein Data Bank under the following accession codes: dATP-inhibited NrdE filament (EMD-9272; PDB 6MWD) and dATP-inhibited NrdE filament (EMD-9293; PDB 6MTY). Source SAXS data underlying Figs. 2a, b, and 3a is provided as a Source Data file. Other data are available from the corresponding author upon reasonable request.

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Author contributions

W.C.T and N.A. designed the research and wrote the manuscript. W.C.T. purified protein and performed SAXS, crystallography, and EM, including data analysis, structure determination, and refinement of X-ray and EM models. F.P.B. purified protein and grew crystals used in this study, F.P.B and A.A.B. performed supporting experiments, and J.P.B. performed crystallographic data processing and refinement of X-ray and EM models. J.S., I.T.K., and J.Z.C. contributed to experimental design and interpretation. J.T.K. and J.Z.C. performed EM data collection and single-particle reconstructions, and J.Z.C. developed software for helical reconstructions. N.A. collected X-ray data, performed research, and led the study. All authors edited the manuscript.

Additional information

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