Molecular interplay of an assembly machinery for nitrous oxide reductase

Christoph Müller,[1,†] Lin Zhang,[1,†] Sara Zipfel, Annika Topitsch, Marleen Lutz, Johannes Eckert,[1] Benedikt Prasser,[1] Mohamed Chami,[3] Wei Lü,[2] Juan Du[2,*] & Oliver Einsle[1,*]

[1] *Institut für Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstrasse 21, 79104 Freiburg, Germany*

[2] *Department of Structural Biology, Van Andel Institute, 333 Bostwick Ave. NE, Grand Rapids, MI 49503, USA*

[3] *BioEM lab, Biozentrum, Universität Basel, Mattenstrasse 26, 4056 Basel, Switzerland*

Emissions of the critical ozone-depleting and greenhouse gas nitrous oxide (N_2O) from soils and industrial processes have increased significantly over the last decades[1-3]. As the final step of bacterial denitrification, the critical greenhouse gas N_2O is reduced to chemically inert N_2 (ref. 1,4) in a reaction catalyzed by the copper-dependent nitrous oxide reductase (N_2OR) [5]. The assembly of its unique [4Cu:2S] active site cluster Cu_Z requires both the ATP-binding-cassette (ABC) complex NosDFY and the membrane-anchored copper chaperone NosL[4,6]. Here we report cryo-electron microscopy structures of *Pseudomonas stutzeri* NosDFY, and its complexes with NosL and N_2OR, respectively. We find that the periplasmic NosD protein features a binding site for a Cu^+ ion and interacts specifically with NosL in its nucleotide-free state, while its binding to N_2OR requires a conformational change triggered by ATP binding. Mutually exclusive complex structures of NosDFY with NosL and N_2OR reveal a sequential metal trafficking and assembly pathway for the most complex copper site known to date. In it, NosDFY acts as a mechanical energy transducer rather than a transporter. It links ATP hydrolysis in the cytoplasm to a conformational transition of the NosD subunit in the periplasm, which is required for NosDFY to switch its interaction partner so that copper ions are handed over from the chaperone NosL to the enzyme N_2OR.

The odorless and non-toxic gas nitrous oxide (N_2O) is formed through biological and abiological processes[7,8], leading to a steady yearly increase in atmospheric concentrations by 0.2–0.3 % [9,10]. N_2O acts as an ozone-depleting substance; its global warming potential exceeds the one of carbon dioxide 300-fold and it has been designated the most critical anthropogenic emission in the 21st century[2]. Microorganisms detoxify N_2O to N_2 as the final step of denitrification[11]. This reaction is catalyzed exclusively by the enzyme nitrous oxide reductase (N_2OR) [5], and a major factor in atmospheric N_2O release is the limited ability of microbial N_2O reducers to process the high flux of nitrogen applied as fertilizers[12]. Consequently, harnessing the performance of N_2OR for agricultural or bioremediatory applications is of high interest and requires detailed understanding of the enzyme and its maturation. The copper-dependent metalloenzyme N_2OR is assembled in the periplasm of Gram-negative bacteria or the extracytoplasmic space of Gram-positives[5]. Beside the [4Cu:2S] Cu_Z cluster it also contains the mixed-valent dicopper electron transfer center Cu_A (Extended Data Fig. 1a), making it the most complex copper enzyme known to date.

Various eukaryotic and prokaryotic enzymes utilize the transition metal copper in processes involving oxygen transport, electron transfer or redox catalysis[13], but its substantial cytotoxicity, detrimental effect on iron-sulfur cluster metabolism and proclivity to generate reactive oxygen species necessitates tight intracellular homeostasis and regulation[14]. N_2O reducers circumvent the risks associated with intracellular copper by assembling Cu_A and Cu_Z entirely outside of the cytoplasm, although apo-N_2OR is exported already in a folded state *via* the *Tat* pathway[15]. This strategy, however, leads to new complications, including in particular the absence of reducing equivalents and high-energy compounds such as nucleoside triphosphates in the periplasm. Clade I N_2O reducers such as *Pseudomonas stutzeri* encode N_2OR together with the machinery required for its assembly and operation in a gene cluster with the core composition *nosRZDFYL* (Extended Data Fig. 1b,c, see Supplementary Discussion). Following the structural gene for N_2OR, *nosZ*, the four genes *nosDFYL* almost invariably form a transcriptional unit centered on the ABC transporter NosFY. As the largest class of primary active transporters, ABC transporters fulfil a broad range of functions[16]. Their common architecture comprises two nucleotide-binding domains (NBD, here

NosF) and two transmembrane domains (TMD, here NosY) [17]. Some bacterial exporters further interact with accessory proteins to build up sophisticated transport machineries[18,19], and the NosD protein was suggested to act as such in conjunction with NosFY[20]. Notably, NosDFY is essential for Cu_Z maturation, but not for that of Cu_A. A mutant of NosDFY defective in ATP hydrolysis resulted in dysfunctional N_2OR that lacked the Cu_Z cluster[21]. Thus, the ABC transporter complex was hypothesized to export sulfide or another [S] species required for Cu_Z assembly from the cytoplasm[22], as it is not readily available in the oxidizing periplasmic environment. An actual cargo molecule for NosDFY yet remains to be identified and a periplasmic sulfur source for Cu_Z maturation cannot be ruled out. The clade I *nos* machinery involves at least two further components, NosR and NosL. The integral membrane protein NosR is a putative quinol oxidase that provides electrons for the reduction of N_2O by N_2OR and was suggested to play a role in regulating the transcription of other *nos* genes (Extended Data Fig. 1b) [5,23]. The membrane-anchored lipoprotein NosL is a Cu chaperone required for Cu_Z assembly[24] and was suggested to reside in the outer membrane of Gram-negative bacteria[25,26]. However, the postulated locations of NosL in the outer membrane and NosDFY in the inner membrane seem to contradict the interplay of the components of this protein machinery during the assembly of N_2OR in the periplasm. To understand the molecular basis of N_2OR maturation, we have produced and characterized the NosDFY complex and studied its interaction with both NosL and N_2OR by cryo-electron microscopy (cryo-EM), revealing an unprecedented, sequential assembly line for the copper sites of the periplasmic enzyme, driven by ATP hydrolysis in the cytoplasm.

Structure of nucleotide-free NosDFY

P. stutzeri NosDFY was produced in *E. coli* as a stable five-subunit complex, $NosDF_2Y_2$, and was isolated by chromatographic methods following solubilization of the membrane fraction (Extended Data Fig. 2a). Single-particle analysis yielded structural models for the detergent- (Fig. 1a, Supplementary Fig. 3) and nanodisc-reconstituted (Supplementary Fig. 4) proteins to 3.3 Å and 3.6 Å resolution, respectively, with no manifest differences between the two strategies for

solubilization and reconstitution. The $NosF_2Y_2$ heterotetramer formed the core of the complex (Fig. 1b), from which the 45 kDa NosD protein protruded into the periplasm as an elongated β-helix with structural similarity to proteins of the carbohydrate-binding and sugar hydrolases (CASH) family[27]. The principal axis of NosD tilted away from the two-fold axis relating the NosFY pair, breaking the symmetry of the molecule (Extended Data Fig. 3). At the NosD-NosY interface, the C-terminus of NosD folded into three α-helices (*h*I–III) that were in part located within the membrane, wedged tightly into the NosY dimer. Accordingly, the insertion of bulky amino acids at either end of helix *h*III rendered NosDFY inactive (Extended Data Fig. 10b,c). NosD also had a cation bound to its C-terminus that was modeled as Mg^{2+} according to its ligand environment and octahedral coordination geometry (Extended Data Fig. 5). As the longest and most C-terminal helix *h*III was nearly perpendicular to the transmembrane helices of NosY (Fig. 1c), its binding displaced several of these, inducing a pronounced asymmetry within the NosY dimer (Extended Data Fig. 6a-c). The topology of NosY matches the type II exporter fold[28] (type V ABC system fold, Extended Data Fig. 6d) [29], with six TM helices and three kinked helices (5a, 5b, 5c between TM 5 and 6) that only enter the outer leaflet of the membrane (Extended Data Fig. 6d,e). The high resolution obtained for the β-helical NosD indicates structural rigidity and a tight binding to NosY, which in turn attained an unusually wide, outward-open conformation without any direct interaction between the two NosY protomers (Fig. 1b) [30]. An inter-subunit gap with a minimal internal diameter of 5 Å spanned the entire distance from the NBDs to the NosD-NosY interface. Despite a similar membrane topology, the nucleotide-free conformation of NosFY is thus very different from that of most other type-II exporters, such as the bacterial O-antigen transporter Wzm/Wzt (Extended Data Fig. 6d) [31]. Interestingly, however, a similar conformation was found for human ABCA1 that also contains a large periplasmic domain, albeit without any similarity to NosD (Extended Data Fig. 7e-g, see Supplementary Discussion) [32]. Both NosDFY and ABCA1 also feature a C-terminal extension of the NBDs. Such domains are not unprecedented[33,34], and in keeping with the terminology for ABCA1 we use the designation ‘R domain’ (Fig. 1b), although an actual **r**egulatory function is not confirmed for the eukaryotic transporter[32]. Resolving the R

domain in the electron density map of nucleotide-free NosDFY required a focused classification that yielded three conformations (Supplementary Fig. 3, 4). The two R domains of the functional $NosF_2$ dimer interacted tightly but were flexible with respect to the remainder of the transporter. Beside the NosD protein, only the R domain connects the two NosFY units in the wide-open, nucleotide-free conformation and is – at least in this state – essential for the integrity of the entire complex.

ATP-bound NosDFY realigns its periplasmic subunit

To characterize an ATP-bound state of NosDFY, we generated an E154Q(NosF) variant that replaced the catalytic glutamate 154 with inactive glutamine. This single-point variant showed strongly reduced ATP-hydrolyzing activity (Extended Data Fig. 7b-d), and when expressed in the context of the recombinant *nos* operon it led to a dysfunctional N_2OR that lacked the active site Cu_Z cluster[21]. This inactive E154Q variant trapped NosF in an ATP-bound state (Fig. 2a, Supplementary Fig. 5), as had been reported for other ABC proteins[35,36]. The binding of ATP led to a substantial closure of the $NosF_2$ dimer (Fig. 2a, Supplementary Fig. 2). This motion was directly transduced to the NosY dimer to close the membrane-spanning gap (Fig. 2d), which eventually induced a complex conformational change of NosD in the periplasm that can be described by three primary modes of rotation, increasing the distance of the HMM motif from the membrane by 8 Å (Fig. 2a). First, the ATP-induced closing of the NosY dimer (Fig. 2e) caused the C-terminal helices to rotate counterclockwise by about 50°, pushing most of helix *h*III out of the membrane (Fig. 2f, red arrow). Second, the Mg^{2+}-binding loop fully relocated from one protomer to the other, causing the release of the cation (Fig. 2d,e). Third, the N- and C-terminal attachment points of the NosD polypeptide (blue dot at N350 and red dot at K420 in Fig. 2d,e,f) followed along, leading to a 60° rotation (Fig. 2f, green arrow) of NosD and a 30° displacement of the long axis of the β-helix (Fig. 2f, blue arrow). The hinge region consisting of the transient Mg^{2+} site and the C-terminal helices thus achieves the conversion of the linear closure of the TMDs into a rotation of NosD. Notably, this action is driven by ATP hydrolysis at NosF and therefore constitutes the

mechanical coupling of an extracellular process to a cytoplasmic source of energy, emerging as a key event for N_2OR maturation by NosDFY.

NosD harbors a transient Cu^+ binding site

Multiple sequence alignments of NosD orthologs revealed a highly conserved array of amino acids comprising residues H207, M209 and M231 (Fig. 2c, Extended Data Fig. 7a). It is located at the approximate center of the β-helix (violet disc in Fig 2a), complemented by the less conserved M279 located on a flexible loop nearby (Fig. 2b,c). The nature and arrangement of this HMM motif is in line with a transient binding site for copper, with soft sulfur ligands that favor Cu^+ over Cu^{2+} but using the more weakly coordinating methionine rather than cysteine to make the association more easily reversible. While the exact environment of the binding site is quite distinct, these principles are also observed in copper chaperones such as CopZ[37] or human Ccs[38]. As a prerequisite for the following variant study and to lift ambiguities present in literature, we investigated the requirement for NosL and NosDFY for the assembly of Cu_A and Cu_Z at low (25 µM) and high (250 µM) copper concentrations (Fig. 2g). We find that NosL is dispensable at high Cu concentrations, but that no maturation of Cu_Z occurs in the absence of NosDFY, independent of the amounts of copper provided (Fig. 2g). We proceeded to vary the HMM triad and its surrounding residues by site-directed mutagenesis and co-expressed individual alanine mutations with tagged *nosZ* in *E. coli*[21]. The occupancy of the Cu-centers of the enzyme was then quantified by UV/vis spectroscopy, verifying functional Cu_Z assembly in N_2OR by its characteristic absorptions at 550 nm and 650 nm in the reduced state (Fig. 2h). These bands were absent in the two variants H207A and M209A and were markedly decreased in variant M231A (Fig. 2h). In contrast, a M279A substitution still yielded spectroscopically intact N_2OR. Our results support an essential role of the HMM triad as a transient Cu binding site essential for the assembly of Cu_Z in N_2OR. The extended loop containing M279 seemed to form a lid-like structure, possibly shielding a bound Cu^+ ion at the HMM triad from the environment. Residue M279 is not conserved among NosD sequences. Its removal decreased the yield of mature N_2OR by about 70% at low external Cu concentrations (25µM) but

had no discernible phenotype at high Cu (250 µM, Extended Data Fig. 10d), suggesting a function for the lid loop in stabilizing Cu bound to NosD. Unlike Cu_Z, the dinuclear Cu_A, characterized by prominent bands at 485, 530 and 795 nm in the oxidized state, was assembled in N_2OR co-produced with any of the NosD variants (Fig. 2h), consistent with a Cu_A assembly that is independent of NosDFY, but only if sufficient copper is provided in the medium (Fig. 2g) [21].

Nucleotide-free NosDFY fetches Cu^+ from NosL

In a recent crystal structure of holo-NosL from *Shewanella denitrificans* we identified a novel, heterobimetallic Cu,Zn site with a structural Zn^{2+} ion and the exchangeable Cu^+ as a cargo[39]. To assess a possible role of NosL as a Cu donor to NosD, we co-produced *P. stutzeri* NosL with the native NosDFY complex, as well as with the variant carrying the E154Q(NosF) mutation that is locked in an ATP-bound state. A stable NosDFYL complex was obtained with nucleotide-free NosF, but not with the ATP-bound variant (Extended Data Fig. 2). NosDFYL was isolated in DDM and reconstituted into GDN micelles and MSP nanodiscs to be characterized by cryo-EM at 3.3Å (nanodiscs) or 3.04 Å resolution (GDN micelles) (Fig. 3a, Supplementary Fig. 6,7). The location of NosL in the complex was immediately apparent (Supplementary Fig. 7a), and its N-terminus was resolved up to the lipid attachment site at C24(NosL) that located precisely at the membrane interface, while the lipid anchor itself was not resolved (Fig. 3b). The arrangement clarified that NosL is not actually located in the outer membrane as previously proposed[5], but in the outer leaflet of the cytoplasmic membrane (see Supplementary Discussion). Furthermore, a truncated version of NosL with the signal sequence including the lipidation site at C24 replaced by the *E. coli* pelB leader did not yield a stable association of NosL with NosDFY. The tethering of NosL to the inner membrane thus is a prerequisite for forming a NosDFYL complex, although the lipid anchor itself does not interact with NosY.

When bound to NosL, the conformation of NosDFY was unchanged from its nucleotide-free state, including the presence of the Mg^{2+} cation at the C-terminus of NosD (Fig. 3b). The soluble part of NosL was in exclusive contact with NosD, and the interface of both subunits involved the

Cu^+-binding site of the chaperone, as well as the HMM triad on NosD, the putative transient metallation site (Fig. 3c). The structure thus provides direct evidence for copper transfer between the two proteins, and the resolution of the cryo-EM map was sufficient to unambiguously locate the zinc and copper ions on holo-NosL (Fig. 3d, Extended Data Fig. 4a,b). In line with a structural role, the Zn^{2+} cation of the chaperone retained the exact position observed in the holo-structure of *Sd*NosL[39], while the Cu^+ ion had moved from its proposed resting state in holo-NosL (Fig. 3e) *via* the activated form observed in the *S. denitrificans* crystal structure (Fig. 3f) by more than 5 Å, essentially completing the transfer to NosD. In its new position the metal was coordinated by the three residues of the HMM triad, H207, M209 and M231, and retained a single ligand from NosL, residue M50 (Fig. 3g). Note also that the removal of C48(NosL) that bridges the Zn^{2+} and Cu^+ ions in the holo form of the chaperone, led to non-functional NosL and thus to the absence of Cu_A and Cu_Z at low Cu concentrations (Extended Data Fig. 10). The lid loop with residue M279 had relocated and was disordered in the cryo-EM map. Hence, the complex formation of holo-NosL with NosDFY alone was sufficient to initiate and largely complete copper transfer to NosD, but both interaction partners remained stably connected. Only when the binding of ATP to NosF then initiates the closure of the NosY dimer, does NosD then rotate its Cu-binding site away from the membrane (Fig. 2a,d), and the tethered NosL is invariably released.

ATP-bound NosDFY delivers Cu^+ to apo-N_2OR

The direct observation of copper transfer from NosL to NosD suggested that NosD itself, rather than NosL, might serve as the metal donor for the Cu_Z site in apo-N_2OR, the NosZ protein. We therefore investigated the formation of a NosZDFY complex in analogy to the interaction study with NosL, i.e. by co-producing NosZ with either wild-type NosDFY or with the E154Q(NosF) variant that stably binds ATP. This time only the E154Q(NosF) variant, but not the wild type, formed a stable NosZDFY complex that was isolated and reconstituted into micelles for cryo-EM single particle analysis (Extended Data Fig. 2, 10b, Supplementary Fig. 8). In the resulting 3.8 Å resolution structure the dimeric apo-N_2OR was tightly bound to NosD (Fig. 4a), precisely

juxtaposing the dimer interface of N_2OR with the HMM triad of NosD. In this arrangement, the N_2OR dimer is close to, but not in contact with the cytoplasmic membrane. The lid loop of NosD, where M279 is located, was in an open conformation to expose the HMM triad (Fig. 4b). Both Cu_A and Cu_Z were fully deplete of copper as expected (Fig. 4c, Extended Data Fig. 4c,d), as the locked E154Q(NosF) variant cannot interact with NosL to be metallated. In the structure, the Cu_A and Cu_Z sites both are located less than 20 Å away from the HMM motif. The Ca^{2+}-binding loop of N_2OR was in an ordered conformation with the divalent cation bound. This was remarkable insofar as we showed earlier that assembly of the copper sites in N_2OR must precede the binding of Ca^{2+}, because the cation stabilizes a loop that connects the β-propeller and cupredoxin domains of different monomers and thereby prevents access for copper ions to the two metal sites of the enzyme[15]. However, the ordered Ca^{2+}-binding loop in the complex structure was in direct and extensive contact with NosD (Fig. 4c). Thus, while Ca^{2+} binding to N_2OR prevents the *in vitro* assembly of Cu_A with Cu^{2+} ions[15], it may not impede the physiological process *via* NosD to the same degree. The complex structure also readily explains why the observed mode of N_2OR binding to NosD is only accessible in the ATP-bound state: Upon conversion to the nucleotide-free form, NosD rotates its copper-binding site back towards the membrane, which would cause bound N_2OR to clash with the membrane and must therefore lead to the release of the apo-enzyme (Fig. 4d).

A full cycle of Cu transfer

In the interplay of the three components, the ATP-driven rotational movement of NosD controls the interaction with its partners NosL and N_2OR (Fig. 5). Copper-loaded NosL can only bind to NosDFY in the nucleotide-free state (**1**), in which the Cu-binding site on NosD faces towards the membrane, allowing the transfer of Cu^+ from NosL to NosD (**2**). The subsequent binding of ATP to NosF triggers a rotation of NosD that the membrane-tethered NosL cannot follow (**3**), leading to its release (**4**). In this conformation, NosD can now interact with N_2OR through the very same interface, transferring its copper cargo to the metal sites of the enzyme (**5**). ATP-hydrolysis in NosF then returns NosDFY to its nucleotide-free open conformation, and the movement of the N_2OR

dimer towards the membrane will eventually force its release (**6**) and free up the Cu-binding site at the HMM triad on NosD to be loaded with another metal cation from NosL. In either direction, the release of the respective interaction partner is triggered mechanically through the rotational motion of NosD, and the structures of the complexes of NosDFY and its partners show in high detail how the ATP-driven distortion of NosD enables the stepwise transfer of single Cu ions from the mononuclear chaperone NosL to eventually assemble the tetranuclear Cu_Z cluster. The ABC-transporter NosDFY thus plays the unusual role of a transmembrane energy converter that dynamically promotes the association and dissociation of the nascent enzyme to its copper donor at NosD, repurposing a primary active transport protein into an ATP-powered lever that works across a boundary separating two very distinct cellular compartments.

The maturation process of the metal centers of N_2OR consists of six sequential single-ion transfers from NosL *via* NosD to form the Cu_A and Cu_Z centers of only one half of the N_2OR dimer. While our data outline an exact route for this process, the formation of Cu_Z additionally requires the accurately timed insertion of two sulfides into the tetranuclear cluster. For this reason, and contrary to Cu_A, the Cu_Z site cannot be assembled *in vitro* by an addition of excess copper, but the actions of NosL in concert with NosDFY are sufficient to build the [4Cu:2S] cluster, suggesting that NosDFY may act as an exporter for a sulfur species[5]. Our work now does not identify any such cargo for NosDFY, but it also does not rule out this additional functionality of the system. The wide cleft between the NosY subunits of NosDFY in its outward-open state consists exclusively of hydrophobic residues, where we observed electron density features that can be modeled as lipid or detergent molecules. Many other type-II exporters act on large, hydrophobic substrates, so that one way to rationalize a possible transport function of NosFY for Cu_Z assembly is that it shuttles (or flips) a sulfur unit linked to a larger, hydrophobic carrier molecule. How a sulfur species might be attached to such a carrier and how it could be released into the periplasm and inserted into N_2OR remains to be elucidated.

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Abbreviations TMD, transmembrane domain; NBD nucleotide-binding domain; ATP, adenosine-5'-triphosphate; AMPPNP, β-γ-imidoadenosine-5'-triphosphate

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FIGURE LEGENDS

Figure 1 | Three-dimensional structure of *P. stutzeri* NosDFY in the nucleotide-free state. **a**, Cryo-electron microscopic reconstruction of the $NosDF_2Y_2$ complex in a GDN micelle viewed parallel to the membrane. **b**, Cartoon representation of the atomic model for NosDFY with the position of the membrane indicated (cytoplasmic face blue). **c**, Interface of NosD with the NosY dimer in the membrane. Three C-terminal helices, *h*I–III, of NosD induce a distinct asymmetry in the NosY protomers. A Mg^{2+} cation is bound at the C-terminus of NosD.

Figure 2 | Conformational dynamics of NosDFY and Cu binding to NosD. **a**, Nanodisc-reconstituted NosDFY with nucleotide-free NosF (left) and the ATP-bound E154Q(NosF) variant (right). ATP triggers a conformational switch (center) that rotates NosD and the Cu-binding HMM motif. **b**, Surface representation of NosD, with the HMM motif and the lid loop (orange). **c**, The HMM motif with H207, M209 and M231, and the lid loop with M279. **d**, Top view of the NosY dimer with the C-terminus of NosD in the nucleotide-free state. Blue and red dots denote G349 and K421, respectively, that connect to the β-helical part of NosD. Arrows indicate the dimer closure upon ATP binding. **e**, The NosY dimer in the ATP-bound state. The Mg^{2+}-binding loop shifts to the other protomer. **f**, Rotational modes in the conformational change of NosD. The C-terminal helices *h*I-III of NosD (red arrow) tilt when the NosY dimer closes, effecting a rotation (green) and tilt (blue) of the β-helix that repositions the HMM motif (violet disc, (a)). **g**, At low [Cu], NosL is required for maturation of both Cu_A (above) and Cu_Z (below). At (unphysiological) 250 µM Cu, Cu_A is assembled without NosL, but Cu_Z is not. *n*=3 independent experiment. Bars are represented as mean values ± SD. **h**, Wild type N_2OR shows contributions of Cu_Z with bands at 550 nm and 650 nm. Upon oxidation, the dinuclear Cu_A site also becomes visible, resulting in a purple species. In a H207A(NosD) variant of N_2OR, no signal for Cu_Z is observed, but Cu_A is intact due to excess Cu in the medium. In the M209A variant, Cu_Z is also absent, while M231A contains residual Cu_Z.

For the M279A lid loop variant, cluster integrity and protein yields are close to wild type. Solid lines: oxidized state, dashed: ascorbate-reduced, dotted: dithionite-reduced.

Figure 3 | Nucleotide-free NosDFY receives Cu^+ from NosL. **a**, Cryo-EM map for NosDFYL viewed parallel to the membrane. **b**, Cartoon representation of the NosDFYL model. The EM map resolves the N-terminal C24 that serves as attachment site for the lipid anchor. **c**, The NosL/NosD interface shows that NosL is in exclusive contact with NosD. **d**, In a close-up of the metal-binding sites of both proteins, a Zn^{2+} ion remains bound to NosL, but the Cu+ ion is already transferred to NosD, binding to the HMM motif (Fig. 2). **e**, An earlier analysis of holo-NosL from *S. denitrificans* indicated a (modeled) resting state with a Zn, Cu site with a metal distance of 2.8 Å. **f**, In a crystal structure of the same protein, a histidine (H104) from a neighboring monomer had engaged the Cu^+ ion, leading to a partial displacement with a metal distance of 3.5 Å that was interpreted as a first activation step. **g**, In line with this, *P. stutzeri* NosL in the present work has largely transferred Cu^+ to NosD (metal distance 7.9 Å), but still coordinates the metal via residue M50.

Figure 4 | Apo-enzyme interaction with the ATP-bound state of NosDFY. **a**, Cryo-EM map for the complex of N_2OR/NosZ with the (NosF)E154Q variant of NosDFY that is locked in the closed state. The atomic model (right) highlights that in this conformation apo-N_2O reductase is not in direct contact with the membrane. **b**, Surface representation of NosD in the complex (see Fig. 2b). The lid loop is in an open conformation, the HMM motif (violet disc) is accessible. **c**, Detail of the interface region of NosD (green) and N_2OR (purple) seen from the membrane towards the periplasm. The HMM motif of NosD is located close to the sites of the copper centers Cu_A and Cu_Z. In the background, a Ca^{2+}-binding loop stabilizes the two NosZ monomers. **d**, The conformational change of of NosDFY upon ATP hydrolysis leads to a rotation of the HMM motif towards the membrane. Bound N_2OR would severely clash with the membrane in this conformation

(bottom, red line), so that the maturation complex effectively will eject the bound enzyme, freeing the HMM motif to receive another Cu^+ ion from NosL.

Figure 5 | Model for copper trafficking from NosL *via* NosDFY to N_2OR. The cryo-EM analysis of NosDFY outlines all steps of the transfer of a Cu^+ ion from the chaperone NosL to apo-N_2OR, the NosZ protein. Nucleotide-free NosDFY (**1**) can form a tight complex with the lipoprotein NosL (**2**) that immediately transfers its Cu^+ cargo to the HMM triad (violet disc). ATP binding then changes the conformation of NosD (**3**), releasing NosL (**4**). In the ATP-bound state, N_2OR can dock to NosD and receive Cu^+ (**5**). The conformation of NosDFY then reverts upon ATP hydrolysis and N_2OR is sheared off (**6**), preparing the maturation machinery for the transfer of the next metal ion.

METHODS

Design and cloning of expression vectors for NosDFY and its complexes

The entire *nos* gene cluster containing the *nosRZDFYLtatE* genes was amplified from plasmid pPR6hE as described previously[21,23]. For the co-production of NosDFY, the *nosD* gene was cloned into a modified pET22a(+) vector (Novagen) containing its original signal sequence and a C-terminal Strep-Tag, while *nosFY* was cloned into a pET28a(+) vector (Novagen) without affinity tag. Higher yields of the complex were obtained with this two-plasmid system, but after the establishment of production and isolation protocols, subsequent work then was continued with single-plasmid expression systems. For the production of the NosDFYL complex, all four genes were cloned into a modified pET30a(+) vector (Novagen), retaining the original signal sequences and adding a C-terminal Strep-Tag to NosL. The NosZDFY complex was produced using a two-plasmid system: pET22::*nosZ*(P) contained a C-terminal Strep-Tag sequence at *nosZ*, and pET30-nosDFY(P) contained an N-terminal His-Tag plus the E154Q exchange at *nosF*. Introduction of affinity tags and single point mutations was carried out by site-directed mutagenesis using the polymerase chain reaction[40].

Recombinant production and purification of proteins

Chemically competent *E. coli* BL21 (DE3) C43 cells were transformed with one (NosDFYL) or two plasmids (NosDFY, NosZDF^{E154Q}Y), depending on the desired protein. NosDFY and NosDFYL were produced in TB medium supplemented with 100 μg·mL^{-1} kanamycin, NosDFY in TB medium with 100 μg·mL^{-1} kanamycin and 50 μg·mL^{-1} ampicillin, and NosZDF^{E154Q}Y in 2×YT medium with 100 μg·mL^{-1} kanamycin and 50 μg·mL^{-1} ampicillin. The cells were cultivated at 37 °C to an OD_{600nm} of 2.0 or 0.5 for TB and 2×YT medium, respectively. After induction with 1 mM of isopropyl-β-*D*-1-thiogalactopyranoside, the cells were further grown for 3-5 h at 20°C and subsequently harvested by centrifugation. Cell pellets were resuspended in a lysis buffer containing 50 mM Tris/HCl at pH 8.0, 0.5 M NaCl and 10% (*v/v*) of glycerol and fractured by sonication

(Emerson Electric, 20 min total pulse time, 3 s pulse, 7 s pause, amplitude 70%). The cell lysate was cleared from cell debris by centrifugation at 30,000×*g* and the supernatant further centrifuged for 45 min at 300,000×*g* to obtain the membrane fraction. The membranes were resuspended in TBSM buffer (20 mM Tris/HCl buffer at pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$), homogenized, and stirred for 1 h at 4°C after adding *n*-dodecyl β-*D*-maltoside (DDM) to a final concentration of 1% (*w/v*). Insoluble remains were separated by centrifugation at 100,000×*g* for 30 min and the supernatant was loaded onto a 5 ml StrepTactin Superflow cartridge (IBA) at low flow over night. NosDFYL was eluted directly from the StrepTactin column in TBSM buffer containing 0.02% (*w/v*) DDM and 0.1% (*w/v*) *D*-desthiobiotin (IBA). NosZDF^{E154Q}Y was eluted onto a 5 ml HisTrap column (Sigma Aldrich) that was equilibrated in TBMS containing 0.02% (*w/v*) of DDM and 10 mM imidazole. Both proteins were further purified by size exclusion chromatography (Superdex S200, 10/300, GE Healthcare) equilibrated in TBSM buffer containing 0.02% (*w/v*) DDM, respectively. The peak fractions were concentrated to 5-10 mg·mL^{-1} in a 100 kDa cut-off centrifugal filter (Sartorius). For cryo-EM analysis of NosDFY reconstituted with the sterol-derived detergent glyco-diosgenin (GDN), the detergent was gradually exchanged during the wash step on the affinity column. Recombinant N_2OR was produced by co-expression of C-terminally Strep-tagged pET22*nosZ* and untagged pET30*nosDFY* in *E. coli* BL21 (DE3) C43 cells and purified as described previously[21]. Mutations in *nosD*, *nosF* and *nosZ* were introduced *via* site-directed mutagenesis. [40] Aliquots were flash-frozen in liquid nitrogen and stored at −80°C.

Preparation of nanodiscs

For the formation of lipid nanodiscs, the membrane scaffold protein (MSP) 1D1 was produced and purified as described previously[41]. *E. coli* polar lipids (Avanti) were dried in a nitrogen stream, resuspended in TBSM buffer containing 0.5% (*w/v*) DDM and solubilized by sonication. Protein solubilized and purified with DDM, MSP1D1 and the preconditioned lipids were mixed at a molar ratio of 1:2:100 (protein:MSP1D1:lipids). The mixture was incubated for 1 h on ice, then for 1 h with activated Bio-Beads at 4°C, under slow rotation of the tube. The Bio-Beads were then

replenished, and the mixture was further incubated overnight. Bead removal was followed by centrifugation for 10 min at 15,000×*g*. The sample was loaded onto a Superose 6 10/300 or Superdex S200 10/300 column (GE Healthcare) and the reconstituted protein was then concentrated and used for grid preparation or ATPase assays.

ATPase activity assays

The activity of ATP hydrolysis was determined by a molybdenum blue assay[42]. Samples were prepared in volumes of 30 µL in TBSM buffer containing 1.5 µM of protein, 0.1-10 mM ATP and 0.02% (*w/v*) DDM (for detergent samples only) and incubated for 10 min at 30°C. The reaction was quenched by the addition of 30 µL 12% (*w/v*) SDS solution. Then 60 µL of 0.5% (w/v) ammonium molybdate and 3% (*w/v*) of ascorbic acid in 1M HCl were added and incubated at room temperature for 5 min before adding 90 µL of 2% (*w/v*) sodium citrate and 2% (*w/v*) of acetic acid. The mixture was then incubated at 37°C for 10 min and subsequently the absorption at 850 nm was determined to quantify the formation of molybdenum blue, $(NH_4)_3[P(Mo_3O_{10})_4]$.

Electron excitation spectroscopy

The assembly of the Cu_Z and Cu_A sites in recombinant N_2OR was assessed by electron excitation (UV/vis) spectroscopy of protein purified *via* size exclusion chromatography. Spectra were recorded with 0.1 mM protein in a buffer containing 20 mM Tris/HCl at pH 8.0 and 150 mM NaCl over a scan range of 200-900 nm using a USB 4000 Spectrometer with USB-ISS-UV/VIS Integrated Sampling System (Ocean Optics). Stoichiometric amounts of potassium ferricyanide were used for oxidation of the Cu_A center. Stoichiometric amounts of sodium ascorbate were used to fully reduce Cu_A, followed by addition of a stoichiometric amount of sodium dithionite to partly reduce Cu_Z.

Cryo-EM grid preparation and data acquisition

Samples reconstituted in either detergent or nanodiscs were directly used after size exclusion chromatography. Protein reconstituted in nanodiscs was supplemented with 0.5 mM of (1H, 1H,

2H, 2H-perfluorooctyl)-β-*D*-maltopyranoside (FOM) prior to grid freezing to improve particle distribution on the grid. NosDF^{E154Q}Y was incubated with 2.5 mM ATP for 10 min on ice, and NosDFY complexed with an ATP analog was incubated with 10 mM AMPPNP for 10 min at room temperature prior to grid freezing. All cryo grids were prepared with 2.5 µL of protein sample using a Vitrobot Mark III (Thermo Fisher) at 100% humidity and a temperature of 16°C. The samples, at a concentration of 7.5-15 mg·mL^{-1}, were applied to glow-discharged Quantifoil (1.2/1.3 or 2/1) 300-mesh gold grids, incubated for 5 s, blotted for 1.5 s with filter paper and flash-frozen in liquid ethane cooled by liquid nitrogen. Data sets were recorded using SerialEM[43] on a 300 kV Titan Krios transmission electron microscope (Thermo Fisher). The NosFY (GDN), NosDFY (GDN) and DFY-AMPPNP (GDN) datasets were recorded on a Gatan K2 summit direct electron detector in super-resolution counting mode with a binned pixel size of 0.513 Å·px^{-1}, 40 frames, and an exposure time of 8 s, with a total dose 49.6 e^{-}·Å^{-2}. The NosDFY (nanodisc), NosDF^{E154Q}Y-ATP (nanodisc) and NosDFYL (nanodisc) datasets were recorded using a Gatan K3 summit direct electron detector at a binned pixel size of 0.324 Å·px^{-1}, 50 frames and an exposure time of 1 s, resulting in a total dose of 50 e^{-}·Å^{-2}. Cryo grids of NosZDF^{E154Q}Y (DDM) were prepared with 3 µL of protein sample using a Vitrobot Mark VI (Thermo Fisher) at 90% humidity and a temperature of 10°C. The samples, at a concentration of 7.5-15 mg·mL^{-1}, were applied to glow-discharged Quantifoil R2/1 300-mesh copper grids, incubated for 5 s, blotted for 2 s with filter paper and flash-frozen in liquid ethane cooled by liquid nitrogen. The dataset was recorded using SerialEM on a Glacios transmission electron microscope (200kV X-FEG optics) equipped with a Gatan K3 detector at a pixel size of 0.87 Å·px^{-1}, 30 frames with a total dose of 50 e^{-}·Å^{-2}.

Image processing

The detailed workflow for the data processing procedures is summarized in the respective Supplementary Figures. In general, the raw movie stacks were motion-corrected using MotionCor2[44] or RELION 3.1[45,46], and the per-micrograph defocus values were estimated using Gctf[47] or CTFFIND4.1[48]. Particle picking was initially done manually or using the Laplacian-of-

Gaussian blob detection in RELION, followed by a template-based picking algorithm from Gautomatch or RELION 3.1. The particles were subjected to one or two rounds of 2D classification, where for the datasets of NosDFY-AMPPNP, NosDF^{E154Q}Y-ATP and NosZDF^{E154Q}Y it was necessary to use the "ignore CTF until first peak" option. Reasonable 2D classes were used for a 3D classification with 60 Å low-pass filtered reference maps that were created in either RELION or cryoSPARC[49]. The best 3D classes were then selected for an initial 3D refinement, followed by Bayesian particle polishing[50] and CTF refinement. Detergent and nanodisc subtraction was achieved for all datasets by generating a mask from the initial model that was expanded by 6 pixels with a soft edge of 6 pixels. The particle set was subjected to 3D classification without image alignment or directly used for 3D refinement, yielding slightly improved maps for most datasets. At this stage the density for the R-domain appeared blurry, indicating flexibility. To trace a possible conformational ensemble of the R-domain, partial signal subtraction followed by masked 3D classification and refinement[51,52] was performed for the datasets of NosDFY (GDN), NosDFY (nanodisc) and NosDFYL (nanodisc). Partial signal subtraction was achieved by using a mask created from atomic models of the domain and were expanded by 6 pixels with an additional soft edge of 6 pixels. The following 3D classification without image alignment resulted in several classes of well-defined R-domains at different relative positions. The particles of the best classes were reverted to original particles and subjected to separate 3D refinements. In the dataset of NosDF^{E154Q}Y-ATP (nanodisc), conformational heterogeneity was observed for the NosD subunit. Consequently, a focused, masked 3D classification was carried out, where excellent density for NosD was resolved in one class (Supplementary Figure 5). For the dataset of NosDFYL (nanodisc), signal subtraction for the NosL subunit followed by masked 3D classification and refinement resolved the N-terminal helix that attached to the lipid anchor at residue C24 (Supplementary Figure 6). Post-processing of all maps was carried out with soft masks generated from the subsequent 3D refinement maps in RELION by expanding the mask by 6 pixels and adding a soft edge of 6 pixels. Density modification and

local resolution map refinement of NosZDFY (DDM) datasets was performed in PHENIX[53]. No symmetry was imposed on any of the data sets.

Model building and refinement

A first model of NosDFY was built *de novo* into the densities of the consensus refinement and the corresponding post-processed map of the GDN dataset using Coot[54]. The R-domain was first built into the NosFY map and subsequently fitted into the NosDFY map. The models of NosDFY, AMPPNP-bound NosDFY, ATP-bound NosDF^{E154Q}Y and NosZDFY were generated by rigid-body real-space refinement in PHENIX[53]. The placement of AMPPNP was performed manually and the binding-site was adjusted according to the density. The maps of ATP-bound NosDF^{E154Q}Y clearly indicated the binding of two molecules of ATP, but the low resolution did not allow for atomic interpretation of the binding site. Instead, the nucleotide-binding site of *E. coli* MalK (Protein Data Bank: 3RLF), solved by X-ray crystallography, was placed into the density and adjusted manually and by real-space refinement in PHENIX. Refinement statistics as well as the validation report generated by MolProbity[55] are listed in Extended Data Table 1. Figures were prepared with PyMOL (Schrödinger LLC), ChimeraX[56], and Origin Pro (OriginLabs).

Data availability

The atomic coordinates of the NosDFY, NosFY and NosDFY-AMPPNP in GDN as well as NosDFY and NosDF^{E154Q}Y-ATP, NosDFYL and NosZDF^{E154Q}Y-ATP in DDM micelles have been deposited with the Protein Data Bank at *http://www.pdb.org*. Several models were uploaded for the different states of the R-domain. The three-dimensional cryo-EM reconstructions of the masked maps post-processing have been deposited with the Electron Microscopy Data Bank (EMDB). Accession numbers of the individual models in the PDB and EMDB are listed in Extended Data Table 1.

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EXTENDED DATA

Extended Data Figure Legends

Extended Data Figure 1 | The nitrous oxide reductase (*nos*) system. **a**, The copper-dependent enzyme N_2OR assembles a composite active site at the homodimer interface, consisting of the mixed-valent [$Cu^{1.5+}$:$Cu^{1.5+}$] electron transfer center Cu_A in one monomer, and the unique, tetranuclear Cu_Z cluster with composition [4Cu:2S] in the other monomer. Substrate access and binding occurs between both clusters. [6] **b**, The Nos system employs the ABC transporter NosFY in conjunction with the periplasmic protein NosD, as well as the dedicated copper chaperone NosL, which was described until now as an outer membrane lipoprotein, while the ascribed role of NosD was in sulfur delivery to N_2OR. In addition, the *nos* operon contains the putative quinol oxidase NosR that requires the NosX protein (in *P. stutzeri* replaced by AbpE[57]) and may transfer electrons to N_2OR. Alternatively, a soluble cytochrome c_{552} was suggested as physiological electron donor to the enzyme[58]. **c**, The *nos* machinery of *P. stutzeri* is encoded in a gene cluster of the structure *nosRZDFYLtatE*.

Extended Data Figure 2 | SEC profiles in DDM micelles and nanodiscs of NosDFY and $NosDF^{E154Q}Y$ and the complexes NosDFY with NosL and $NosDF^{E154Q}Y$ with NosZ, together with the corresponding analyses by SDS-PAGE. All purifications were performed at least three times with highly reproducible outcomes. The figure shows representative SEC profiles and SDS PAGEs.

Extended Data Figure 3 | Structural Details of NosDFY. **a**, NosD, colored from blue at the N-terminus to red at the C-terminus (marked). Helices *h*i, *h*II and *h*III interact with NosY. **b**, The ATPase domain NosF with the C-terminal R domain. **c**, The transmembrane domain NosY, with the second protomer colored in white. **d**, In the NosF dimer, the R domains are crossed, providing

additional stability. **e**, In the nucleotide-free state, the R-domain was found in an ensemble of conformations that could be individually refined and revealed a high degree of structural flexibility. **f**, Superposition of NosD (green) with its closest structural relative, the CASH family protein DFA-IIIase (PDB 5ZKS, r.m.s.d. = 2.68 Å). **g**, NosF (lightorange) and the related ABC domain MalK of the *E. coli* maltose importer (PDB 3RLF, r.m.s.d. = 2.05 Å). Note that while MalK contains an additional C-terminal domain, it shows no relevant similarity to the R domain. **h**, NosY (red) and the TMD of the Wzm/Wzt O antigen transporter (PDB 6M96, r.m.s.d. = 6.0 Å).

Extended Data Figure 4 | Exemplary electron density maps NosD. **a**, The maps section shows a stereo representation of the electron density map around the HMM motif of NosD as indicated in the cartoon of the complex. In the ATP-bound state of the E154Q(NosF) variant complex, the HMM motif (residues H297, M209, M231) are metal-free. **b**, Electron density map for the NosDFYL complex in GDN micelles, showing the same region as in (a). Zn^{2+} binds to NosL and Cu^{+} is coordinated by the HMM motif of NosD. **c**, As the NosZDFY complex was formed with the E154Q(NosF) variant that produces a NosDFY complex that cannot receive Cu from NosL, the metal sites of the apoenzyme are vacant. Electron density map at the Cu_A site of N_2OR. The structure of the holo-form of the enzyme (right) underlines the absence of the two Cu ions. **d**, Electron density map at the Cu_Z site in the hub of the β-propeller domain of N_2OR. The Cu-replete protein in the same orientation (right) denotes where the tetranuclear cluster should be located. All maps are normalized and contoured at the 5σ level.

Extended Data Figure 5 | The Mg^{2+}-binding site and conformations of NosD. **a**, Stereo representation of the C-terminal Mg^{2+}-binding loop in the nucleotide-free state of the NosDFY complex. The cation is octahedrally coordinated in the region from residues D359 to D367, orienting arginine R360 to tightly interact with the membrane-integral NosY subunit. **b**, Cartoon of NosD bound to the open NosY dimer in the nucleotide-free state of NosDFY. The HMM motif

is indicated by a violet disc. **c**, Cartoon of NosD bound to the closed NosY dimer in the ATP-bound E154Q(NosF) variant.

Extended Data Figure 6 | The transmembrane module belongs to type-IV ABC transporters (type-II exporters). **a-c**, Structural asymmetry in the NosY dimer induced by binding of NosD. Top views, colored by relative root-mean-squared deviations of atom positions. **a**, Nucleotide-free NosFY subcomplex with symmetric protomers. **b**, Nucleotide-free NosDFY complex. Major distortions are observed on the periplasmic side of the right protomer, in TM helix 5 and the absence of subhelix 5c (green circle in (a)) due to structural disorder. **c**, ATP-bound structure of $NosDF^{E154Q}Y$. As the NosY protomers close, TM helix 5 moves outward, but the structural asymmetry remains, including the disorder of helix 5c in the right protomer. **d**, Intramembrane modules for *P. stutzeri* NosY, *H. sapiens* ABCG5/G8, *H. sapiens* ABCA1 and *A. aeolicus* Wzm in cartoon representation. For each system, one subunit of the TMDs is colored from blue (N-terminus) to red (C-terminus). **e**, Following the topology of NosY, the individual transmembrane helices and the small reentrant helices 5a, 5b and 5c are shown in their relative orientation within the membrane with representative EM density maps, contoured at the 5 σ level.

Extended Data Figure 7 | Properties of NosDFY complexes and related transporters. **a,** Sequence alignment for the HMM motif in NosD proteins. The histidine and two methionine residues are among the most highly conserved in NosD. **b**, ATP-hydrolytic activity of Nos(D)FY(L) complexes. Substrate dependence of ATPase activity for NosFY, NosDFY, NosDFYL and the E154Q(NosD) variant of the NosDFY complex in DDM micelles. **c**, Comparison of ATPase activity at [ATP] = 1 mM for protein preparations reconstituted in DDM micelles or MSP nanodiscs. Note that reconstitution of the E154Q(NosD) variant in nanodiscs did not yield a good SEC profile and was therefore not considered successful (Extended Data Fig. 2). n=3 technically independent samples. Bars are represented as mean values ± SD. **d**, Data table for

(b). **e**, The human transporter ABCA1, or cholesterol efflux regulator protein (CERP). Cryo-EM structure of human ABCA1, depicted in an open state[32]. The entire transporter is a single polypeptide, and the extensive extracellular domain is composed of insertions into both transmembrane subunits. In the structural model, the prominent R domains are not swapped between the two halves of the transporter. **e**, Human ABCA1 as predicted by AlphaFold2[59]. The transmembrane domains are highly similar to the closed state of NosDFY in the NBDs and TMDs. Note that in this model the R domains are swapped, as is the case in NosDFY. **f**, Conformational differences in the extracellular domain of ABCA1 between cryo-EM structure and AlphaFold2 prediction, in front and top view, respectively. As in NosD, the domain undergoes a rotational motion upon closure of the transporter that is most pronounced in the apical domain.

Extended Data Figure 8 | Variant studies on the interactions of NosD. **a**, Residue C48(NosL) bridges the Zn^{2+}- and Cu^{+}-binding sites of the chaperone. Its removal renders the chaperone inactive, as evidenced by the failure to mature either Cu site of N_2OR at low external Cu. **b**, Residue P393(NosD) is in the NosD-NosY interface, at the N-terminus of helix *h*III. Its replacement for a bulky Trp largely, but not fully impairs the assembly of Cu_Z. Note that the maturation assay was carried out at high Cu concentrations to have direct Cu_A assembly by Cu^{2+} as a positive control. **c**, Residue V407(NosD) marks the C-terminal end of helix *h*III. Disturbing this interaction renders NosDFY fully non-functional. **d**, The lid loop of NosD with residue M279(NosD) is not highly conserved among NosD proteins. Accordingly, its deletion impairs, but not prevents Cu_Z maturation at low copper concentration (middle). At high Cu concentrations, N_2OR maturation is intact, pointing towards a role of the lid loop in stabilizing Cu bound to NosD.

Extended Data Table 1 | Cryo-EM data collection, refinement, and validation statistics.

	DFY_GDN			FY_GDN	DFY_GDN_AMPPNP	DFY_nano				$DF^{E154Q}Y$_nano + ATP	
	consensus	R domain k7	R domain k9	consensus micelle subtracted	consensus micelle subtracted	consensus	R domain k1+k2	R domain k3	R domain k4+k5	consensus	best NosD
PDB accession # EMDB accession #	7O0Y 12683	7O10 12685	7O11 12686	7O0Z 12684	7O12 12687	7O13 12688	7O14 12689	7O16 12691	7O15 12690		7O17 12692
microscope	Titan Krios					Titan Krios					
detector	Gatan K2 direct electron detector					Gatan K3 direct electron detector					
magnification	130,000					130,000					
voltage (kV)	300					300					
exposure (e^{-}/Å^2)	1.24					1.0					
defocus range (μm)	-1.0 to -2.5					-1.0 to -2.5					
pixel size (Å)	1.026					0.648					
number of movies	6,405			4,839	11,050	7,204				7,317	
imposed symmetry	C1			C1	C1	C1				C1	
initial particle number	1,497,395			1,854,589	2,159,966	1,762,074				1,218,640	
final particle number	410,321	65,429	60,309	743,288	573,516	337,471	130,946	68,188	138,319	106,305	20,977
resolution (Å)	3.3	3.6	3.7	3.7	3.7	3.6	3.8	4	3.8	4.3	4.5
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Refinement											
resolution (Å)	3.2	3.7	3.9	3.8	4.1	3.7	3.7	3.9	3.7	4.4	4.5
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
r.m.s. deviations in											
bond lengths (Å)	0.004	0.007	0.008	0.006	0.006	0.008	0.008	0.006	0.008	0.009	0.012
bond angles (°)	0.778	0.901	0.969	1	0.868	0.942	0.9	0.861	0.898	1.194	1.229
Validation											
MolProbity score	1.59	1.87	2.04	2.15	1.9	1.78	1.79	1.8	1.8	2.41	2.8
clash score	4.59	8.66	11.08	6.02	9.76	6.28	6.35	7.14	6.48	21.71	22.26
poor rotamers (%)	0.52	0.7	1.22	3.01	0.26	0.78	0.7	0.35	0.78	0.44	2.82
Ramachandran plot											
favored (%)	94.87	93.99	93.86	92.79	94.2	93.39	93.12	93.86	93.18	88.71	87.26
allowed (%)	5.06	5.94	6.07	7.21	0.26	6.55	6.82	6.07	6.75	11.22	12.67
disallowed (%)	0.07	0.07	0.07	0	0.07	0.07	0.07	0.07	0.07	0.07	0.07

Extended Data Table 2 *(continued)* | Cryo-EM data collection, refinement, and validation statistics.

	DFYL_nano				DFYL_GDN	ZDF^{E154Q}Y_DDM
	consensus	R-domain k1	R-domain k3	Mem. anchor k5	consensus	consensus
PDB accession #	7OSG 13050	7OSF 13049	7OSI 13052	7OSJ 13053	7ZNQ 14813	7QBA 13885
Microscope	Titan Krios				Titan Krios	Glacios
Detector	Gatan K3 direct electron detector				Gatan K2	Gatan K3
Magnification	130,000				165,000	46,000
Voltage (kV)	300				300	200
Exposure ($e^-/Å^2$)	1.24				1.25	1.67
Defocus range (μm]	-1.0 to -2.5				-1.0 to -2.0	-1.0 to -2.0
Pixel size (Å)	0.648				0.82	0.87
Number of movies	7,829				9,949	2,453
Symmetry imposed	C1				C1	C1
Initial particle number	947,940				1,978,836	855,511
Final particle number	165,858	37,715	33,900	31,587	420,836	114,710
Resolution (Å)	3.3	3.8	3.8	3.8	3.04	3.76
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Refinement						
Model resolution (Å)	3.3	3.7	3.7	3.8	3.15	3.78
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5
R.m.s.d.						
Bond lengths (Å)	0.009	0.007	0.007	0.007	0.004	0.006
Bond angles (°)	1.123	1.107	0.992	1.094	0.888	1.065
Validation						
MolProbity score	1.62	1.62	1.73	1.66	1.63	2.11
Clashscore	4.26	4.26	5.22	4.72	4.36	16.35
Poor rotamers (%)	0.89	0.89	0.6	0.97	0.22	0.58
Ramachandran plot						
Favored (%)	93.68	93.74	93.01	93.59	93.82	94.10
Allowed (%)	6.32	6.26	6.99	6.35	5.88	5.53
Disallowed (%)	0	0	0	0.06	0.30	0.37