SUPPORTING INFORMATION

The Crystal Structures of Bacillithiol Disulfide Reductase Bdr (YpdA) Provide Structural and Functional Insight into a New Type of FAD-Containing NADPH-Dependent Oxidoreductase

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Section S1. Expression, Purification and Characterization

Expression and Purification of Bc YpdA, Sa YpdA, and Sa YpdA G10A mutant

pET-22b(+) plasmids containing the genes for Bc YpdA (BC1495, Bc ATCC 14579, restriction enzymes NdeI and BamHI)\(^1\), Sa YpdA (SACOL1520, Sa COL, restriction enzymes NdeI and HindIII) or Sa YpdA G10A (SACOL1520, Sa COL, restriction enzymes NdeI and HindIII) (GenScript) were transformed into competent *Escherichia coli* One Shot\(^\text{TM}\) BL21 (DE3) cells (Invitrogen, Thermo Fischer Scientific). Cells containing either of the three plasmids were grown in Terrific Broth medium containing 100 \(\mu\)g/mL ampicillin. Protein expression was induced by adding isopropyl \(\beta\)-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM at \(\text{OD}_{600\text{nm}} = 0.7-0.9\), and the cultures were incubated for 12-16 hours at 20°C with vigorous shaking before cells were harvested and frozen at -20°C. Cells were thawed and dissolved in 100 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 \(\mu\)g/mL DNase, cOmplete Protease Inhibitor Cocktail (Roche) in a 1:4 cell weight to buffer ratio and lysed by sonication. Alternatively, Sa YpdA, as well as Sa YpdA G10A, were dissolved in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 \(\mu\)g/mL DNase, cOmplete Protease Inhibitor Cocktail (Roche), prior to cell lysis. The suspensions were centrifuged at 48,000g and the lysates were cleared from nucleic acids by streptomycin sulfate (2.5 %) precipitation, followed by centrifugation at 48,000g. From the lysates, these Tag-free proteins were precipitated with ammonium sulfate ((NH\(_4\))\(_2\)SO\(_4\)) to final concentrations of 0.25 g/mL and 0.22 g/mL for Bc and Sa YpdAs, respectively, and centrifuged at 39,000g. Proteins were dissolved in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and desalted using a HiTrap Desalting column (GE Healthcare). Desalted proteins were applied to a HiTrap HP Q column and eluted with linear or step-wise 0-0.5 M KCl or NaCl gradients. As a final polishing step, protein used for crystallization experiments were purified on a Superdex 200 or Superdex 200 Increase column (GE Healthcare) in 50 mM Hepes, pH 7.5, 100 mM KCl. All chromatographic steps were performed using an Äkta purifier FPLC system (GE Healthcare). Protein fractions were pooled, concentrated in Amicon Ultra-15 filter units (10 or 30 kDA MWCO, Merck-Millipore), flash-frozen in liq N\(_2\), and stored at -80°C.

Preparation of Se-Methionine Derivatives

The L-selenomethionine (Se-Met) derivatives of Bc and Sa YpdA were expressed and purified in a manner similar to that of the wild type proteins, with the following modifications. Cells were grown at 37°C in M9 minimal medium supplemented with L-methionine (50 mg/L) until the \(\text{OD}_{600\text{nm}}\) had reached 1, harvested, and resuspended in fresh M9 minimal medium without methionine. Cells were further incubated at 37°C until the addition of lysine, phenylalanine, and threonine (100 mg/L of each); isoleucine, leucine, and valine (50 mg/L of each); and L-selenomethionine (50 mg/L), prior to induction with 0.5 mM IPTG (at \(\text{OD}_{600\text{nm}} = 1\)) and incubation for 16 hours at 20°C with vigorous shaking before harvesting and freezing of the cell paste. Cell lysis and protein purification was performed as described for the wild type proteins.

Dynamic Light Scattering (DLS) and Native Polyacrylamide Gel Electrophoresis (PAGE) Analyses of Protein Oligomerization

In order to estimate the size and molecular weight of the purified Sa and Bc YpdA proteins, oligomeric states of the YpdA proteins were investigated by DLS (Zetasizer Nano), in 50 mM Hepes, pH 7.5, 100 mM KCl, at 25°C and with 50 \(\mu\)M protein concentrations, in three replicates each. In addition, protein samples of both Sa and Bc YpdA (2 \(\mu\)M and 3 \(\mu\)M, respectively) were...
analyzed on Native PAGE (NativePAGE™ Novex® 4-16% Bis-Tris Protein Gels, Thermo Fisher Scientific).

UV-vis Spectroscopy of Native and Mutant YpdAs

UV-vis absorption spectroscopy was performed on 10 µM purified Sa YpdA, Bc YpdA, and Sa YpdA G10A mutant proteins (Agilent Cary 60) to examine the flavin-bound states of the native YpdAs and the apo-form of the G10A mutant. Concentrations were assessed from the absorbance at 453 nm ($\varepsilon_{453nm} = 11.5$ mM$^{-1}$cm$^{-1}$) (native proteins) and 280 nm ($\varepsilon_{280nm} = 32.3$ mM$^{-1}$cm$^{-1}$) (mutant protein).

Section S2. Activity Measurements

Preparation of BSSB Substrate

As bacillithiol disulfide (BSSB) is not commercially available, an oxidation of reduced BSH was performed, as described by Hamilton and coworkers. In short, prior to enzymatic assays, a solution of NH$_4$HCO$_3$ was added to reduced BSH (Jema Biosciences) dissolved in water at room temperature and stirred with exposure to air for 1 h, flash-frozen in liq N$_2$, and stored at -80°C. Oxidation of BSH to BSSB was verified with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Enzymatic Assays of BSSB Reduction by Sa and Bc YpdAs

The reduction of BSSB by NADPH-dependent YpdAs was investigated through spectroscopic determination of the reductase activity. Activity was verified through following the decrease in absorption caused by the oxidation of NADPH at 340 nm. Enzymatic assays performed in the presence of dioxygen were executed at 25°C in 20 mM Tris, 1.25 mM EDTA, pH 8.0, 200 µM NADPH, and varying concentrations of BSSB (0.25-10 µM BSSB), followed by the addition of 1 µM purified Sa or Bc YpdA after 1 min, and spectra were collected every second for 1000-1200 seconds at 340 nm (Agilent Cary 60). Due to the oxygen sensitivity seen for both Sa and Bc YpdAs, enzymatic assays were also performed under anaerobic conditions in a glovebox (Plas-Labs Anaerobic Chamber 855-AC and an Agilent 8453 diode-array UV-vis spectrophotometer). The Sa YpdA G10A mutant was used as a control, proving the importance of Gly10 in binding of the FAD cofactor, crucial for enzymatic activity. All solutions were degassed on a Schlenk line before transfer to the glovebox. Buffers and stock solutions were sparged with argon for minimum 2 hours in vented vials, and protein samples were subjected to 5-6 cycles of evacuation and refilling with argon. Assays were performed at 25°C in 20 mM Tris, 1.25 mM EDTA, pH 8.0, 240 µM NADPH, with the addition of 12.5 µM purified Sa YpdA, Bc YpdA or Sa YpdA G10A mutant, followed by the addition of 50 µM BSSB, and spectra were collected every second for 250 seconds at 340 nm. Prior to addition of BSSB, reactions were run for 2 min to allow for the reduction of trace amounts of dioxygen by YpdA, in the presence of NADPH. All assays were performed in replicates.

Section S3. Protein Crystallography

Protein Crystallization

All initial crystallization screening was performed with a Mosquito crystallization robot (SPT Labtech). Conditions that identified initial hits were further optimized by systematic
optimization using the sitting drop vapor diffusion method. Native Sa YpdA crystals (57 mg/mL) were obtained with condition A7 (Sa YpdA 1) or A11 (Sa YpdA 2) from the Morpheus crystallization screen (Molecular Dimensions) (A7: 0.03 M magnesium chloride, 0.03 M calcium chloride, 0.1 M MOPS/HEPES-Na, pH = 7.5, 10% w/v PEG 4000, 20% v/v glycerol or A11: 0.03 M magnesium chloride, 0.03 M calcium chloride, 0.1 M bicine/Trizma, pH = 8.5, 10% w/v PEG 4000, 20% v/v glycerol). Native Bc YpdA crystals (22 mg/mL) were obtained with condition B8 from the JCSG-plus crystallization screen (Molecular Dimensions) (0.2 M magnesium chloride hexahydrate, 0.1 M Tris, pH = 7.0, 10% w/v PEG 8000), and briefly soaked in cryoprotectant solution containing mother liquor and 20% ethylene glycol. Diffraction-quality crystals for the Se-Met variant of Bc YpdA (25 mg/mL) were grown in condition G3 from the Morpheus crystallization screen (Molecular Dimensions) (0.02 M sodium formate, 0.02 M trisodium citrate, 0.02 M sodium potassium L-bicine/Trizma, pH = 8.5, 10% w/v PEG 4000, 20% v/v glycerol). All crystals were grown at room temperature, and flash-frozen in liq N2 prior to data collection.

Crystal Data Collection, Processing, and Refinement

Diffraction data were collected at MAX IV, Lund, Sweden, on beam line BioMAX (Sa YpdA and Se-Met Bc YpdA) and at ESRF, Grenoble, France, on beam line ID23-1 (Bc YpdA) through MXCuBE3 3 and iSPyB 4 at 100 K. The Se-Met Bc YpdA diffraction data set was collected at 0.976252 Å, a few eV above the theoretical Se absorption K-edge. Diffraction data were indexed and integrated with iMosflm 5 (Bc YpdA), auto-processed with EDNA 6 and XDS 7 (Sa YpdA), and autoPROC 8 and XDS (Se-Met Bc YpdA), and scaled and merged with Aimless in the CCP4 package 9.

Although BLAST sequence alignment searches against the PDB databases indicated hits with up to 30% sequence identity, extended molecular replacement trials with different search models were not successful in solving the structure. This was most likely due to the number of subunits in the asymmetric unit, 4 and 8 combined for Bc and Sa, respectively, with the possibility of relative movements of the FAD and NADPH binding domains.

The Se-Met Bc YpdA structure was solved by single-wavelength anomalous dispersion (SAD) experiment. Matthews coefficient analysis indicated four molecules in the asymmetric unit with a Matthews coefficient of 2.6 Å3/Da and solvent content of 53.2%, indicating that the anomalous signal was significant to about 3.8 Å, so the data set was scaled to 3.5 Å with an anomalous redundancy of 6.6. To solve the structure with SAD, CRANK2 11 was used through CCP4 online using SFTools, SHELX, SHELXD, REFMAC5, PEAKMAX, MAPRO, Solomon, Multicomb, Parrot, and Buccaneer 12-18. The solved structure contained four molecules in the asymmetric unit, and initially refined to an R-factor of 36%. This initial model was used as the starting model for the higher resolution native Bc YpdA data set of 1.6 Å resolution. After an initial refinement with REFMAC5 further automatic model building was performed with ARP/wARP 19-21 through the CCP4i. This was followed by several cycles of refinement with phenix.refine 22 in the Phenix suite 23 and model building in Coot 24. TLS refinements was performed with each of the four chains constituting a TLS group. During the refinement also a PDB_REDO run was performed 25. Model validation was performed using MolProbity 26. All structure figures were prepared with PyMOL (Schrödinger, LLC).

The Sa YpdA 1 and 2 structures were solved independently through molecular replacement (MR) using the Bc YpdA structure as a starting model. Eight molecules in the asymmetric unit would give a Matthews coefficient of 2.8 Å3/Da and solvent content of 55.6%. To solve the full
structure with eight molecules in the asymmetric unit with Phaser \(^27\) through CCP4i, one had to search for one copy of the Bc YpdA tetramer, and four copies of the Bc YpdA monomer. This was followed by several cycles of refinement initially with REMACS and subsequently phenix.refine in the Phenix suite, and model building in Coot. Model validation was performed using MolProbity.

For Bc YpdA, all residues have been modelled for chains A and B. For chain C, the C-terminal residue has been excluded, and for chain D, the N-terminal and C-terminal residues have been excluded due to poor electron density. It can additionally be noted that some of the modelled loops have limited electron density. Chains G and H have large areas with poor electron density, show high temperature factors, and are more distorted than the other chains (Table S1). Due to the very limited and unclear electron density in some areas of chain G, residues 159-215 (Sa YpdA 1) and 179-187 + 196-215 (Sa YpdA 2) were not built into the model for chain G. Although chains G and H are disordered, they were independently a solution in MR, and removing them from the structure increased both \(R_{\text{work}}\) and \(R_{\text{free}}\) and was therefore retained in the final structures.

Clear electron density was observed for FAD and modelled in all subunits of both Bc YpdA and Sa YpdAs, although with less clear density in the two more distorted chains G and H in Sa YpdAs (Figure S13A,B). No electron density was observed for NADPH in the Bc YpdA structure where both conformations were observed for the possible gating residue Tyr133 (Figure 3F,G). Both Sa YpdAs showed clear electron density for NADPH in chains C, D and F, which was accompanied with the closed conformation of residues 295-301, and Tyr128 in open conformation with hydrogen bonding to NADPH (Figure 3D,E,F S13B,D,E). NADPH was not observed in chains A, B and E, which was accompanied with the open conformation of residues 295-301, and Tyr128 in the closed conformation (Figure 3D,E,G). In both chains C and F (Sa YpdA 1), one conformation of NADPH was modelled in, while in both chains D and F (Sa YpdA 2), two orientations of NADPH could be observed accompanied by a movement of Phe\(51\) with two alternate conformations (Figure 3H). The orientation of the nicotinamide parts of NADPH have not been fully resolved.

The standard Phenix restraints used for the FAD cofactor were modified for the 1.6 \(\AA\) Bc YpdA resolution structure, to take into account potential X-ray radiation-induced reduction of the FAD cofactor making the isalloxazine ring free to bend along the N5–N10 axis (butterfly bend) \(^28\). The angle of the butterfly bend of the isalloxazine ring was calculated with the psico module in PyMOL, by calculating the angle between the two planes defined by atoms N5, C4X, C4, N3, C2, C10, N10, and N5, C5X, C3, C7, C8, C9, C9A, N10. A slight butterfly bend of average 4.2° was observed for the isalloxazine rings in Bc YpdA, consistent with expected radiation-induced reduction (Figure S13C).

**Crystal Packing**

The Bc YpdA P22,2\(_1\) crystals contained a homo tetramer in the asymmetric unit (Figure S4A), with the monomers having an RMSD value of 1.2-1.3 \(\AA\) relative to each other (Figure S4B). The Sa YpdA P6,22 crystals contained eight monomers (RMSD values 1.2-2.8 \(\AA\) relative to each other, Figure S4E) in the asymmetric unit; a tetramer and two dimers (Figure S4C). The Sa YpdA tetramer is similar to the Bc tetramer as seen from the overlay in Figure S4D (RMSD value of 1.7 \(\AA\)). The closest symmetry equivalent subunits in the Sa YpdA crystal to the two dimers are shown in Figure S4F (yellow and pink), which shows that they also are part of tetramers. Two of the tetramers (Figure S4F, palecyan and yellow) overlay well (RMSD value of 1.3 \(\AA\),
while for the third tetramer (pink), each of the two dimers overlay well (RMSD value of 2.5 Å) with the two dimers of the other tetramers, however, overlaying one dimer results in the second dimer to be shifted 15 Å relative to the others (Figure S4G). Therefore, the Sa YpdA P6,22 crystal also contains tetramers, however, most likely due to crystal packing, one of the tetramers had to adopt by slightly sliding the two dimers relative to each other (Figure S4G). PDBPISA was used to analyze the buried surface area of the YpdA multimers. The ABCD, E,F, and G,H tetramers have a surface area of ~5300 Å² with a buried area of ~16000 Å², which suggests a stable tetrameric structure in solution (Table S3). The tetramers have a higher percentage of buried area than the individual dimers, except the dimer CD with fully bound NADPH.

**Comparison of the Active Site of Selected Flavoprotein Disulfide Reductases**

The electron flow for most flavoprotein disulfide reductases (FDRs) are from NADPH/NADH positioned on the re-face of the isoalloxazine ring, through the FAD and to a cystine or a single Cys on the si-face (Figure S14B,E,F,G,H). The active Cys residues on the si-face are also accessible to substrate or other Cys/SeCys-residues for further reaction (Figure S14). Low M, TrxRs are an exception, where the electron transfer only occurs on the re-face through a rotation of the NADPH domain relative to the FAD domain. The electrons go from NADPH to the FAD, then NADPH is rotated away so the cystine comes close to the FAD, and the cystine is reduced (Figure S14C,D). YpdA does not have a conserved cysteine within 3-4 Å of the isoalloxazine ring nor that is accessible by a substrate. However, the channel above the isoalloxazine ring on the re-face is large enough to accommodate BSSB, and the disulfide in BSSB could be in a similar position to the isoalloxazine ring as the cystine in low-M, TrxR FO (flavin oxidizing) for a potential direct reaction (Figures S14D and S12E).

**Section S4. Bioinformatics and Structural Analysis**

**BLAST Search for Homologous Sequences**

The Sa YpdA sequence (Locus tag SACOL1520) was used for a BLAST (Basic Local Alignment Search Tool) search using the NCBI web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searching the reference proteins (refseq_protein) database searching for 20,000 sequences with a threshold E-value of 1e⁻⁰⁵ using the BLOSUM62 matrix. The same search was also performed limiting the search to the different bacterial phyla.

The BLAST search resulted in 3977 bacterial organism hits. The dominating bacterial phylum for Sa YpdA homologous sequences was Firmicutes (1813 organism hits) followed by Bacteroidetes (1425), Proteobacteria (527), Deinococcus-Thermus (93), Actinobacteria (71) and Acidobacteria (49). Of these organisms, Proteobacteria use GSH as the main LMW thiol, and Actinobacteria use MSH, while low G+C Firmicutes and Deinococcus-Thermus contain BSH, and Bacteroidetes and Acidobacteria to a large extent contain Me-BSH. Therefore, Proteobacteria and Actinobacteria were not further studied, while three of the top hits from Firmicutes, Deinococcus-Thermus, Bacteroidetes, and Acidobacteria were subjected to multiple sequence alignments and phylogenetic tree analysis through Jalview. The alignments were performed with Clustal Omega and tree analysis with average distances using the BLOSUM62 matrix. Figure S9 shows that the sequences fall into four clades corresponding to the four phylum classes the sequences belong to. The sequence identity compared to the Sa YpdA sequence was for the other Firmicutes in Figure S9 ~60%, for Bacteroidetes ~44%, Deinococcus-Thermus ~37%, and Acidobacteria ~44%, and all these sequences have been annotated as putative YpdAs in the refseq_protein database. A cysteine in Sa YpdA has been
suggested to function as an active site residue, involved in the reduction of BSSB. This cysteine is found in the canonical FAD binding GXGXXG motif in Firmicutes, however, in Bacteroidetes, this residue is mainly replaced by isoleucine, in Deinococcus-Thermus valine, and in Acidobacteria threonine (Figure S9). Further studies on the proposed YpdAs from e.g. Bacteroidetes, Deinococcus-Thermus, and Acidobacteria need to be performed to reveal if they are YpdAs or if they have other functions.

The putative YpdA orthologs used in this study were from *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Chryseobacterium frigidisolii*, *Fabibacter pacificus*, *Algoriella xinjiangensis*, *Meiothermus lutes*, *Thermus filiformis*, *Deinococcus misasensis*, *Acidobacteria bacterium*, *Candidatus Sulfotelmatobacter sp.*, and *Granulicella sp.*

**Structure Comparison - Structural Alignment Search with DALI (Distance-matrix Alignment)**

A search for similar structures of the YpdA in the Protein Data Bank (PDB) was performed using the DALI protein structure comparison server using the *Bc* YpdA structure as a search template. The most similar monomer structures were flavoprotein monooxygenases (FPMOs), thioredoxin reductases (TrxRs), thioredoxin-like ferredoxin NADP⁺ oxidoreductases (FNRs), dihydrolipoyl dehydrogenases (DLDs), and glutathione reductases (GRs) (Table S2).

The monomer structures of YpdA were compared to the monomer structures FPMO (PDBid 4C5O), TrxR (PDBid 5VT3), FNR (PDBid 3AB1), DLD (PDBid 6CMZ), and GR (PDBid 1GRB) from Table S6, and are compared and overlaid (Figure S6). In addition, the multimeric structures are shown. For TrxR, both the FR (flavin reducing) and FO (flavin oxidizing) (PDBid 1TDF) dimeric states are shown, and for FNR, the two structures of the *Bc* FNRs are shown (PDBid 6GAS and 6GAR). Figures were generated with PyMOL.

Although the NADPH and FAD domains of YpdA are similar to DLD and GR (Figure S2, Table S2), DLD and GR have 100-130 additional C-terminal residues. Therefore, these proteins were not included in the structural sequence alignments generated with DALI (Figure S3). The secondary structure assignments were calculated with DSSP and shown below the sequence. The figure was generated in JalView and colored by % identity.

**Phylogenetic Analysis of YpdA with other Flavin Oxidoreductases in Selected Firmicutes**

Through several BLAST searches the likely sequences of YpdAs, FNRs, TrxRs, and FPMOs in the selected Firmicutes *Staphylococcus aureus subsp. aureus COL*, *Staphylococcus epidermidis* RP62A, *Bacillus cereus ATCC 14589*, *Bacillus anthracis str. ste rene*, and *Bacillus subtilis subsp. subtilis* str. 168 were found. Multiple sequence alignments were performed with Clustal Omega and phylogenetic tree analysis with average distances using the BLOSUM62 matrix (Figure S6). The figures were generated in JalView and the sequence alignments were colored by % identity. The phylogenetic tree analysis shows that the four oxidoreductase types from the five phyla fall into four clades corresponding to the four oxidoreductases types, and that YpdA forms its own clade, indicating that it constitutes a separate type of flavin oxidoreductases (Figure S6).

The locus tags for the sequences used are shown in parentheses for *Sa* YpdA (SACOL1520), *Sc* YpdA (SERP1047), *Bc* YpdA (BC1495), *Ba* YpdA (BAS1405), *Bs* YpdA (BSU22950), *Sa* FNR1 (SACOL2369), *Bc* FNR1 (BC0385), *Ba* FNR1 (BAS0337), *Bc* FNR2 (BC4926), *Ba* FNR2 (BAS4797), *Bs* YumC (BSU32110), *Ba* YcgT (BSU03270), *Sa* TrxR (SACOL0829), *Sc* TrxR (SERP0432), *Bc* TrxR (BCS159), *Ba* TrxR (BAS007), *Bs* TrxR (BSU34790), *Sc* FPMO (SACP0299), and *Sc* FNR2 (SACP0298).
(SACOL2600), Sc FPMO (SERP2384), Bc FPMO (BC3447), Ba FPMO (BAS3253), and Bs FPMO (BSU26640).

**Analysis of Conserved Residues of YpdA with ConSurf**

To evaluate the degree of conservation of residues in YpdA, ConSurf \(^{36-39}\) was run on the Bc YpdA structure. The run was based on the homologue search algorithm HMMER, searching sequences from UniRef90, and multiple sequence alignment with MAFFT. This gave 2266 unique HMMER hits, and ConSurf used a sample of 150 sequences that represented the list of homologues sequences to map the conservation on a 9-bin scale from turquoise (most variable) to maroon (most conserved). The conservation color coding was then mapped onto the Bc YpdA crystal structure and figures were generated with PyMOL.

Phylogenetic analysis on YpdA was performed in Jalview on the 150 sequences selected in the ConSurf runs (a few outlier sequences were not included). Clustal Omega was used for sequence alignment, and average distances in the phylogenetic tree were calculated with BLOSUM62.

The selected sequences fall into three clades in the phylogenetic trees showing that the most homologous sequences to Bc YpdA is found in other Firmicutes, Bacteroidetes and Acidobacteria (Figure S10).

The surface representation of the ConSurf colored YpdA show that the most conserved part of the surface (white-to-maroon) is between the tetramers, which supports the conclusion of YpdA being a biological tetramer (Figure S11).

The most conserved residues (maroon-to-lightmaroon) are found around the FAD cofactor, around the NADPH binding site, and the residues lining the solvent channel spanning the structure in connection with the FAD cofactor.

**Analysis of Channels with HOLLOW**

The potential channel for BSSB binding was generated with HOLLOW \(^{40}\) using a 4 Å probe in cylinder mode between Phe51 and Tyr152(Bc)/Tyr147(Sa), or Lys170(Bc)/Lys165(Sa) and Tyr152. Similarly, the sphere mode was used to look for any small channels around Cys14.

**Modelling/Estimating the Position of BSSB in YpdA**

To show potential BSSB binding sites around the FAD group, BSSB was manually positioned in Coot within the HOLLOW-generated channels and regularized with respect to stereochemical restraints in Coot. To span potential orientations within the whole channel, three molecules of BSSB were fitted. One was positioned with the disulfide bond close to the C4a (C4X, FAD numbering) atom of FAD (5 Å).

**Ligand Interaction Generated with LigPlot.**

To make schematic diagrams of protein-ligand interactions from the protein structure (PDB files), the program LigPlot\(^*\) was used with default parameters to show hydrogen bonds and hydrophobic contacts represented by dashed lines and arcs with spokes radiating toward the ligand atoms they contact, respectively \(^{41-42}\).
Supplementary Figures and Tables:

Figure S1: NADPH consumption by YpdA under aerobic conditions. YpdA consumes NADPH at higher enzymatic rates with BSSB added to the reaction.
Figure S2: Structure comparison. Monomer structures of YpdA and top DALI hits for the FPMO (PDBid 4C5O\textsuperscript{43}), TrxR (PDBid 5VT3, 1TDF\textsuperscript{44}), FNR (PDBid 3AB1\textsuperscript{45}, 6GAR/6GAS\textsuperscript{46}), GR (PDBid 1GRB\textsuperscript{47}), and DLD (PDBid 6CMZ) from Table S2, the monomer structure alignment of FPMO, TrxR, FNR, GR, and DLD with YpdA, and selected multimerization structures of the different proteins. The PDBid is listed for each structure.
Figure S3: Structural alignment of BcYpdA, FPMO (PDBid 4C5O), TrxR (PDBid 5VT3), and FNR (PDBid 3AB1) performed with a DALI search. The secondary structure assignments were calculated with DSSP and shown below the sequence. The figure was generated in JalView and colored by % identity.
Figure S4: Crystal packing. (A) Asymmetric unit of *Bc YpdA* (tetramer). (B) Overlay of the *Bc YpdA* monomers. (C) Asymmetric unit of *Sa YpdA* (octamer). (D) Overlay of the *Bc YpdA* tetramer with the palecyan colored tetramer of the *Sa YpdA* from B. (E) Overlay of the eight *Sa YpdA* monomers from C. (F) The octamer from B added the closest dimer symmetry equivalents of the yellow and pink colored dimers of the *Sa YpdA* octamer. (G) Overlay the three tetramers shown in F with full overlay of the palecyan and the yellow tetramers. The second dimer of the pink tetramer is shifted 15 Å relative to the first dimer compared, as compared to the palecyan and pink tetramers.
Figure S5: Molecular weight estimation of YpdA proteins. Analysis of the oligomerization state of Sa YpdA (36.7 kDa/monomer) and Bc YpdA (36.5 kDa/monomer) performed with (A) DLS, and (B) Native PAGE, indicating that YpdA is a biological tetramer.
Figure S6 continues on next page
Figure S6: Multiple sequence alignment and phylogenetic tree analysis of proposed YpdAs, FMPOs, FNRs, and TrxRs from the selected Firmicutes *S. aureus, S. epidermis, B. cereus, B. anthracis*, and *B. subtilis*. (A) Multiple sequence alignment generated with Clustal Omega through Jalview. The coloring is according to % identity. The sequences are grouped according to the phylogenetic tree in B. (B) Phylogenetic tree calculated with Jalview with average distances using the BLOSUM62 matrix on the sequence alignment A.
Figure S7: Selected structural features in Bc YpdA. (A) The GGGPCG motif. (B) The environment around Cys14. (C) LigPlots* showing the interaction between Cys14 and other residues with the residue name colored according to conservation obtained from ConSurf.
Figure S8: UV-vis characterization of YpdA proteins. UV-vis absorption spectra of 10 µM purified Sa YpdA, Bc YpdA, and Sa YpdA G10A mutant. The spectra show typical features of the oxidized state of the FAD cofactor in Sa YpdA and Bc YpdA (see inset), whereas the YpdA G10A mutant exists in its apo-form.
Figure S9: Multiple sequence alignment and phylogenetic tree analysis. Analysis of selected YpdA homologous sequences form the phyla Firmicutes, Bacteroidetes, Deinococcus-Thermus and Acidobacteria. (A) Multiple sequence alignment generated with Clustal Omega through Jalview. The coloring is according to % identity. The sequences are grouped according to the phylogenetic tree in B. (B) Phylogenetic tree calculated with Jalview with average distances using the BLOSUM62 matrix on the sequence alignment in A. The common GXGPXG motif of YpdA is shown for each clade. The putative YpdA orthologs used were from S. aureus, B. cereus, B. subtilis, C. frigidisoli, F. pacificus, A. xinjiangensis, M. luteus, T. filiformis, D. misasensis, A. bacterium, C. Sulfotematobacter sp., and G. sp.
Figure S10 continues on next page
Figure S10: Multiple sequence alignment and phylogenetic tree analysis of the ~150 sampled sequences obtained from a ConSurf search using the Bc YpdA structure. (A) Multiple sequence alignment generated with Clustal Omega through Jalview. The coloring is according to % identity. The sequences are grouped according to the phylogenetic tree in B. (B) Phylogenetic tree calculated with Jalview with average distances using the BLOSUM62 matrix on the sequence alignment A.
Figure S11: Conservation of residues in YpdA evaluated using ConSurf. Variable residues are colored in turquoise, highly conserved residues are colored in maroon, and figures are generated in PyMOL. The FAD cofactor is shown as sticks and colored with yellow carbon atoms. The degree of conservation of the YpdA residues are represented as surface, lines and/or cartoon in the different panels. (A) Overall view of the YpdA tetramer in different orientations with the conservation shown for one monomer. (B) Monomer view of conserved residues shown in two different orientations.
Figure S12: Cofactor and putative BSSB binding sites. (A) LigPlot∗ showing the FAD bonding site in Bc YpdA and (B) FAD and NADPH binding sites in Sa YpdA. (C) Three LigPlots∗ showing the ConSurf colored conserved residues interacting with the three possible BSSB molecules placed into the HOLLOW-generated solvent channel. (D) Overlay of the three potential BSSB orientations shown with the three tyrosine residues that are 3-4 Å away from the disulfide in BSSB. (E) Three potential BSSB orientations in the HOLLOW-generated solvent channels in proximity to the FAD cofactor.
Figure S13: Cofactor electron density, geometry and interactions. Electron density for the Bc YpdA structure chain A (A) and Sa YpdA 1 structure chain C (B), with the $2F_o - F_c$ map contoured at $1\sigma$ (colored in darkteal/palecyan) and the $F_o - F_c$ maps contoured at $\pm 3\sigma$ (colored in green/red) around the FAD and NADPH cofactors. (C) The refined butterfly bending of the flavin plane from likely X-ray radiation induced reduction. (D) Electron density for cofactors and residues likely involved in gating of NADPH and possibly BSSB, showing a hydrogen bond from Tyr128 to NADPH in the Sa YpdA 1 NADPH-bound state (open conformation). (E) Omit electron density map of NADPH in Sa YpdA 1 chain C contoured at $3\sigma$ (colored in green).
Figure S14: Active site of selected flavoprotein disulfide reductases. For the different structures the first chain is colored in grey and when shown, the second chain in black. Cofactors are represented as sticks and colored by atom type. The carbon atoms are colored in a unique color for each enzyme. The active site of (A) Sa YpdA 1, (B) GR (PDBid 1GRA and 1GRB, NAPDH from the latter), (C) Low-M, TrxR in FR form (PDBid 5VT3), (D) Low-M, TrxR in FO form (PDBid 1TDE and 1TDF, NADPH from the latter), (E) NADH peroxidase (NPX) (PDBid 1F8W and 2NPX, NADH from latter), (F) Coenzyme A-disulfide reductase (CoADR) (PDBid 4EQR), (G) Mercuric reductase (MerA) (PDBid 1ZK7 and 4K7Z, first and second chain, respectively), (H) High-M, TrxR (PDBid 3EAO and 2ZZC, NADPH from the latter).
Table S1: Crystal data collection and refinement statistics.

| Data collection | Bc YpdA Se-Met | Bc YpdA | Sa YpdA 1 | Sa YpdA 2 |
|-----------------|----------------|---------|-----------|-----------|
| X-ray source    | BioMAX, MAXIV | ID23-1, ESRF | BioMAX, MAXIV | BioMAX, MAXIV |
| Detector        | EIGER 16M     | Pilatus 6M | EIGER 16M | EIGER 16M |
| Wavelength (Å)  | 0.976252      | 0.97242  | 1.49379   | 0.946444  |
| Space group     | P22,2,2       | P22,2,2  | P6,22     | P6,22     |
| a, b, c (Å)     | 88.8, 115.3, 147.9 | 88.7, 114.9, 146.7 | 179.3, 179.3, 349.3 | 180.3, 180.3, 350.5 |
| α, β, γ (°)     | 90, 90, 90    | 90, 90, 90 | 90, 90, 120 | 90, 90, 120 |
| Type of data collection | Standard rotation | Helical scan | Standard rotation | Standard rotation |
| Rotation range per image (°) | 0.1 | 0.1 | 0.1 | 0.1 |
| Total rotation range (°) | 360 | 130 | 180 | 90 |
| Exposure time per image (s) | 0.011 | 0.037 | 0.011 | 0.011 |
| Flux (ph/s) / Transmission (%) | n/a / 25% | 2.1 × 10^{12} / 100% | n/a / 50% | n/a / 50% |
| Beam size (μm$^2$) | 50 × 50 | 30 × 45 | 50 × 50 | 50 × 50 |
| Crystal size (μm$^3$) | 30 × 200 × 200 | 20 × 150 × 250 | 75 × 75 × 250 | 30 × 30 × 100 |
| Mosaicity (°)  | 0.26          | 0.19        | 0.08        | 0.05 |
| Resolution range (Å) | 147.9-3.5 (3.8-3.5) | 73.4-1.65 (1.68-1.65) | 49.6-2.9 (2.96-2.90) | 29.9-3.1 (3.18-3.10) |
| Total no. of reflections | 254621 | 791900 | 1328276 | 606005 |
| No. of unique reflections | 19810 | 175671 | 73800 | 61638 |
| R_meas          | 0.184 (0.344) | 0.086 (0.915) | 0.169 (0.580) | 0.125 (0.505) |
| R_merge         | 0.176 (0.330) | 0.076 (0.810) | 0.165 (0.565) | 0.119 (0.480) |
| Completeness (%) | 99.8 (99.3) | 97.8 (97.5) | 99.8 (99.4) | 99.9 (99.9) |
| Anomalous completeness | 99.9 (99.9) | | | |
| Multiplicity    | 12.9 (13.1)  | 4.5 (4.7)   | 18.0 (20.4) | 9.8 (10.3) |
| Anomalous Multiplicity | 6.6 (6.7) | | | |
| $<I/\sigma(I)>$ | 19.5 (11.2) | 10.0 (2.0) | 13.9 (5.5) | 14.3 (4.5) |
| CC$^{1/2}$      | 0.998 (0.993) | 0.989 (0.728) | 0.997 (0.951) | 0.998 (0.938) |
### Table S1 continues

| Refinement statistics | Bc YpdA Se-Met | Bc YpdA | Sa YpdA 1 | Sa YpdA 2 |
|-----------------------|----------------|---------|-----------|-----------|
| $R_{\text{work}}/R_{\text{free}}$ (%) | 19.0/21.8 | 24.3/30.8 | 24.4 /29.1 |
| Mean protein/ligands/solvent isotropic $B$ factor ($\text{Å}^2$) | 41.6/35.1/41.5 | A-F: 46.8/46.0/35.3 | A-F: 114.4/105.2/55.4 |
| Protein assembly in asymmetric unit (AU) | 4 monomers | 8 monomers | 8 monomers |
| Protein residues in gene | 326 | 328 | 328 |
| Total modelled residues in AU | | | |
| - protein residues by chain | A-B:326,C:325,D:324 | A-F,H:323,G:263 | A-F:324;B:321;C:325;D,E:323,G:287,H:316 |
| - ligands | 4 FAD | 8 FAD, 3 NADPH | 8 FAD, 3 NADPH |
| - added waters | 857 | 26 | 24 |
| Matthews coefficient ($\text{Å}^3/\text{Da}$) | 2.28 | 2.80 | 2.80 |
| Solvent content (%) | 45.5 | 55.8 | 55.7 |
| Ramachandran favored/allowed/outliers (%) | 96.9/2.7/0.4 | A-F: 93.8/5.4/0.8 | A-F: 93.4 /5.4 / 1.2 |
| RMSD bond lengths (Å) | 0.006 | 0.009 | 0.003 |
| RMSD bond angles (°) | 0.83 | 1.12 | 0.64 |
| PDB ID | 7A76 | 7A7B | 7APR |
### Table S2: The structures with the highest Z-score for each of the five most similar protein types from the DALI search of the full PDB of BcYpdA.

| Protein | PDBid | RMSD | Z-score | Aligned residues | Number of residues | % sequence identity | Organism                  |
|---------|-------|------|---------|------------------|-------------------|---------------------|--------------------------|
| FPMO    | 4C5O  | 2.8  | 28.9    | 289              | 330               | 19                  | *Stenotrophomonas maltophilia* |
| TrxR    | 5VT3  | 2.4  | 28.1    | 295              | 313               | 19                  | *Vibrio vulnificus*        |
| DLD     | 6CMZ  | 3.3  | 25.0    | 285              | 459               | 18                  | *Burkholderia cenocepacia* |
| FNR     | 3AB1  | 4.6  | 24.9    | 294              | 318               | 22                  | *Chlorobaculum tepidum*    |
| GR      | 1GRB  | 3.3  | 23.9    | 278              | 461               | 19                  | *Homo sapiens*             |

### Table S3: Multimerization analysis with PDBePISA (Proteins, Interfaces, Structures and Assemblies)

| Multimer      | Structure | Surface area Å² | Buried area Å² | Buried/surface % | ΔG^int kcal/mol | ΔG^diss kcal/mol |
|---------------|-----------|-----------------|----------------|------------------|-----------------|------------------|
| EF₂[FAD]₄[NADPH]₂ | Sa       | 53210           | 16320          | 31               | -76.0           | 7.0              |
| ABCD[FAD]₄[NADPH]₂ | Sa       | 52820           | 16380          | 31               | -73.0           | 5.6              |
| ABCD[FAD]₄      | Bc       | 53020           | 15290          | 29               | -62.3           | 9.1              |
| CD[FAD]₂[NADPH]₂ | Sa       | 27620           | 7970           | 29               | -31.9           | 1.8              |
| G₄H₂[FAD]₄      | Sa       | 54550           | 14260          | 26               | -72.1           | 1.0              |
| EF[FAD]₂[NADPH]  | Sa       | 28170           | 6590           | 23               | -31.3           | 2.2              |
| CD[FAD]₂        | Bc       | 28360           | 5480           | 19               | -28.8           | 3.0              |
| GH[FAD]₂        | Sa       | 28930           | 5470           | 19               | -27.4           | -1.3             |
| AB[FAD]₂        | Bc       | 29000           | 5470           | 19               | -27.1           | 2.4              |
| AB[FAD]₂        | Sa       | 28440           | 5160           | 18               | -29.0           | 2.3              |

A-H indicates the chain ID numbering in the different structures

ΔG^int solvation free energy gain upon formation of the assembly

ΔG^diss free energy of assembly dissociation
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