Experimental procedures

Purification of YxiN mutants

YxiN wild-type and mutants were purified as described (*1, 2*).

Preparation of ADP.BeF_x, ADP.MgF_x, and ADP.AlF_x solutions

ADP·BeF_x, ADP·MgF_x, and ADP·AlF_x nucleotides were prepared by mixing stock solutions of the corresponding metal chloride, NaF, and ADP to a final concentration of 16.5 mM ADP, 82.5 mM metal chloride, and 825 mM NaF.

Fluorescence equilibrium titrations

Dissociation constants of YxiN/nucleotide complexes at 20°C were determined in fluorescence equilibrium titrations in a Hitachi F-4500 fluorimeter using the fluorescent ADP analog mantADP (*3*). Mant fluorescence was excited at 360 nm (5 nm bandwidth) and detected at 440 nm (10 nm bandwidth).

The dissociation constants of YxiN/adenine nucleotide complexes were determined in competitive titrations of the mantADP/YxiN complex with ADP·BeF_x, ADP·MgF_x, ADP·AlF_x and ADPNP. Starting concentrations were 1 μM mant-ADP and 10 μM YxiN. The solution of the cubic equation describing the competitive titration was evaluated numerically using the program Scientist (Micromath) to yield the Kd of the respective nucleotide complex as described (*1, 4*).

RNA substrates

The 153mer RNA substrate comprising nucleotides 2483-2635 of the *B. subtilis* 23S rRNA was generated by T7 polymerase *in vitro* transcription as described (*1*).

The 32/9mer minimal RNA substrate comprising hairpin 92 of the 23S rRNA was constructed by annealing a synthetic 32mer and a synthetic 9mer as described (*1, 2*). Unwinding assays were performed with 5 μM RNA and 10 μM YxiN in 50 mM HEPES, pH 7.2, 150 mM KCl, 5 % glycerol, 0.1 mM DTT, 0.1 mg/ml BSA, 5 mM $MgCl_2$ in the presence of 10 mM ATP or ADP, or 5.5 mM ATP analogs at 25°C. To ensure single turnover conditions, a 10-fold excess

of unlabeled 9mer was added as a trap strand. Products were analyzed by native polyacrylamide gel electrophoresis as described (*1, 2*).

Fluorescent labeling and smFRET experiments

Fluorescent labeling of cysteines was performed in 50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.5 mM TCEP at a protein concentration of ~50 μM with a 3-fold molar excess of Alexa488-maleimide (A488, donor) and a 4-fold molar excess of Alexa546-maleimide (A546, acceptor) for 1 h at 25°C. The reaction was stopped by adding 1 mM BME, and free dye was removed by size exclusion chromatography on Bio-Rad Micro Bio-Spin Columns. Labeling efficiencies were determined from absorbance ratios at 493 nm (A488, corrected for A546 contributions) or 554 nm (A546) and 280 nm (protein, corrected for dye contributions).

Single molecule FRET experiments were performed using a home-built confocal microscope as described (*2*). Only fluorescence bursts above a threshold of 100 photons were considered in the analysis. Measured background-corrected fluorescence intensities were corrected for crosstalk (α: donor crosstalk in acceptor channel, β: acceptor crosstalk in donor channel), different quantum yields and detection efficiencies of donor and acceptor fluorescence (γ), and direct excitation of the acceptor (δ), and converted into FRET efficiencies as described (*2*). Measurements were performed at room temperature (25°C) in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$ with 80 pM fluorescently labeled protein (concentration of donor fluorophore), 5 mM ADP or ADPNP, or 2 mM ADP·BeF_x or ADP·MgF_x, and 200 nM 153mer RNA. ADP·BeF_x-, ADP·MgF_x- and ADP-solutions were incubated for 2h at RT with 400 μM glucose and 7U hexokinase prior to the measurements to remove traces of ATP. For conversion of FRET efficiencies into distances, previously determined Förster distances were used (*2*).

References:

1. Karow, A. R., Theissen, B., and Klostermeier, D. (2007) *FEBS Journal 274*, 463-473.
2. Theissen, B., Karow, A. R., Kohler, J., Gubaev, A., and Klostermeier, D. (2008) *Proc. Natl. Acad. Sci. U. S. A. 105*, 548-553.
3. Hiratsuka, T. (1983) *Biochim. Biophys. Acta 742*, 496-508.
4. Thrall, S. H., Reinstein, J., Wohrl, B. M., and Goody, R. S. (1996) *Biochemistry 35*, 4609-4618.