Supplementary Materials and Methods

Protein purification

The purification of *Chaetomium thermophilum* eIF5B(517-858) was done as previously described (Kuhle & Ficner, 2014). The plasmid for the expression of N-terminally His-tagged *cte*IF5B(517-858) (comprising G domain and domain II) was transformed into *E. coli* BL21(DE3) cells (Stratagene) by heat shock. Transformed cells were grown in 1 l cultures of 2YT medium at 37 °C to an OD_{600} of 0.6-0.8, followed by the induction of protein expression with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for an additional 18 h at 16 °C before harvesting. Harvested cells were resuspended in buffer A (40 mM HEPES/KOH (pH 7.5), 500 mM KCl, 10% glycerol, 30 mM imidazole, 2 mM β-mercaptoethanol), lysed using a Microfluidizer (Microfluidics) and clarified by 30 min ultracentrifugation at 30,000 xg. The supernatant was loaded onto two HisTrap columns (GE Healthcare), equilibrated in buffer A. After the elution of bound proteins with a linear gradient of imidazole (30-300 mM), eIF5B-containing fractions were pooled and desalted in buffer B (20 mM HEPES/KOH (pH 7.5), 200 mM KCl, 10% glycerol, 30 mM imidazole and 2 mM β-mercaptoethanol) for subsequent TEV-protease cleavage at 4 °C over night to remove the His-Tag. Uncleaved protein was removed by a second HisTrap in buffer B and the flow-through was pooled and concentrated for the final size exclusion chromatography step on a Superdex 200 column (GE Healthcare), equilibrated in buffer C (10 mM HEPES/KOH (pH 7.5), 50 mM KCl, 3 mM $MgCl_2$ and 1 mM DTT). The absence of copurified guanine nucleotides was confirmed by HPLC. The purified apo eIF5B was pooled, concentrated to 15-20 mg/ml, and finally stored at -80 °C.

Mutant constructs of *cte*IF5B, in which Asp533 (Asp^{MC}) was substituted by alanine (D533A), asparagine (D533N) or arginine (D533R), respectively, were generated by site-directed mutagenesis according to the QuikChange protocol (Stratagene, Instruction Manual, 2006). All mutants were expressed and purified according to the above described protocol for wild-type *cte*IF5B(517-858) (Kuhle & Ficner, 2014).

For ITC experiments *cte*IF5B(517-858) was purified according to the above protocol with the difference that ITC buffer (30 mM HEPES/KOH (pH 7.5), 100 mM KCl, 10% glycerol, 4 mM β-mercaptoethanol, 0.01% tween 20, 2.5 mM $MgCl_2$) was used in the final gelfiltration step.

For GTPase activity assays under different salt conditions, purified *cte*IF5B(517-858) (wild-type or mutant) was desalted in the desired buffer (Tris/HCl (pH 7.5), 3 mM $MgCl_2$, 2 mM DTT and alkali salts at different concentrations) prior to the experiment (see below).

E. coli EF-Ts was expressed with an N-terminal His-tag in *E. coli* BL21(DE3)pLysS cells (Stratagene). Transformed cells were grown in 1 l cultures of LB medium at 37 °C to an OD_{600} of ~0.8, followed by the induction of protein expression with 0.5 mM IPTG. Cells were grown for an additional 4 h at 37 °C before harvesting. Purification of EF-Ts (including the removal of the His-tag) was performed according to the protocol described above for *cte*IF5B(517-858), with the difference that a Superdex 75 column (GE Healthcare), equilibrated in a buffer containing 25 mM Tris (pH 7.5), 30 mM KCl, 70 mM NH_4Cl and 7 mM $MgCl_2$, was used in the final size exclusion chromatography step. Purified protein was flash-frozen in liquid nitrogen and stored at -80 °C.

E. coli EF-Tu was prepared essentially as described previously (Perla-Kajan et al, 2009). The final gelfiltration buffer contained 20 mM Tris/HCl (pH 7.5), 50 mM KCl, 7 mM $MgCl_2$ and 2 mM DTT. GTP-bound EF-Tu was prepared by incubating 100 µM purified EF-Tu with 3 mM GTP, 3 mM phosphoenolpyruvate (Roche), 0.05 mg/ml pyruvate kinase (Roche) and 0.04 µM EF-Ts for 1 h at 37 °C. To remove excess GTP and phosphoenolpyruvate, GTP-bound EF-Tu was desalted on a HiTrap Desalting column (GE Healthcare) at 4 °C in the desired buffer (25 mM Tris/HCl (pH 7.5 at 25 °C), 7 mM $MgCl_2$, 2 mM DTT and alkali salts at different concentrations).

Crystallization and structure determination

The first crystallization trials with *cte*IF5B(517-858) were performed in an optimization screen for the condition under which the original crystals of GTP-bound *cte*IF5B(517-860) had been obtained using a fragment comprising residues 517-970 (0.1 M HEPES/NaOH (pH 7); 13% PEG 4000; 0.1 M NaOAc) (Kuhle & Ficner, 2014). 8, 10 or 12 mg/ml of protein were mixed with GTP, GTPγS or GDPNP at final concentrations of 1, 2 or 3 mM (up to 10 mM for GDPNP in the presence of 10 mM $MgCl_2$) of the respective guanine nucleotides. Crystallization was performed at 4 and 20 °C by vapor diffusion using a 1:1 or 2:1 ratio of protein to reservoir solution. For *cte*IF5B(517-858)·GDPNP no crystals were obtained under any of the tested conditions. Crystals of *cte*IF5B(517-858)·GTP and *cte*IF5B(517-858)·GTPγS grew over night in most conditions, irrespective of the temperature or used guanine nucleotide concentration. In both cases the best diffracting crystals that were finally used for structure determination grew at 20 °C in primitive monoclinic space group $P2_1$.

To find a crystallization condition for *cte*IF5B(517-858) in the presence of potassium, crystallization trials with 10 mg/ml protein were performed in the presence of 2 mM GTPγS using standard screens. Diffraction quality crystals were obtained after two weeks at 20 °C in a condition containing 11% PEG 8000, 6% glycerol, and 50 mM KCl. The crystals used for

structure determination grew in space group $P4_12_12$ and contained two protein-GTPγS complexes per asymmetric unit.

Initial crystallization trials for the Asp^{MC} mutants of *cte*IF5B(517-858) (D533A, D533N and D533R) were performed with 8 mg/ml protein in the presence of 3 mM GTP or GTPγS using fine-screens around the two conditions described above. Only for the D533N mutant high quality crystals in space group $P2_1$ grew over night that could be directly used to determine the structure of *cte*IF5B(517-858)D533N bound to GTP and a Na^+ ion. No crystals were initially obtained for the other mutants D533A and D533R. We then performed microseeding experiments for both mutants, in which crystals of the wild-type protein were destroyed and used as crystallization nuclei to induce crystallization. At protein concentrations of 15 mg/ml and 6 mM guanine nucleotide, this resulted in the formation of thin needle clusters for D533A in conditions containing 0.1 M HEPES/NaOH (pH 7.3), 15% PEG 4000, and 150 mM NaOAc. These were then used in an additional round of microseeding, finally yielding large enough crystals for structure determination. As the wild-type protein, the D533A mutant crystallized in space group $P2_1$ and the structure was finally solved at a resolution of 1.58 Å. In contrast to D533A, no crystals were obtained for D533R in the initial microseeding experiments but only small spherolite-like aggregates which could not be used as seeds. We therefore performed new crystallization trials for the D533R mutant in various commercially available grid screens, in combination with microseeding. This yielded thin, plate-shaped crystals in a condition containing 100 mM MES (pH 6.5), 11% PEG 8000, and 150 mM NaOAc. Using these crystals as microseeds in an optimized screen, we were finally able to obtain *cte*IF5B(517-858)D533R crystals in the presence of GTPγS suited for structure determination. Despite the fact that the D533R mutant crystals were obtained in the presence of Na^+ ions, they grew in space group $P4_12_12$ as did those obtained in the presence of K^+. The structure of *cte*IF5B(517-858)D533R could finally be solved at a resolution of 2.75 Å.

Based on the observations for the *cte*IF5B mutants, additional crystallization trials were performed with wild-type *cte*IF5B(517-858) in the presence of GDPNP and microseeds. In the fine-screens, microseeding resulted in the formation of large spherolytes, which, however, did not yield any crystals when used as seeds themselves.

For the structures of wild-type cteIF5B(517-858) bound to GTP or GTPγS and Na^+, X-ray diffraction data were collected at P13 beamline at PETRA III (EMBL, Hamburg). For the structures of wild-type cteIF5B(517-858) bound to GTPγS and K^+ and all Asp^{MC} mutants, X-ray diffraction data used for structure determination were collected at beamline ID23-1 at ESRF (Grenoble). For all structures, the phase problem was solved by molecular replacement using the program PHASER (McCoy et al, 2007) with the original structure of *cte*IF5B·GTP as search model. Structures were refined to reasonable R-values and stereochemistry using the program PHENIX (Adams et al, 2010). Data collection and refinement statistics are summarized in Table 1 and Supplementary Table S2.

Structure factors and coordinates for the two aEF1A structures were obtained from the protein data bank (PDB: 3AGJ, 3VMF). The structures were refined using the program PHENIX (Adams et al, 2010). Manual model rebuilding was performed against electron density maps in Coot (Emsley et al, 2010). Figures were prepared using Pymol (http://www.pymol.org).

Isothermal Titration Calorimetry

When ΔH is plotted against the temperature, the slope of the fitted line directly represents the ΔC_p of the binding reaction (Jelesarov & Bosshard, 1999; Prabhu & Sharp, 2005). ΔC_p can be used as an estimate for the change in solvent accessible surface area (ΔASA) upon complex

formation as it was found to be proportional to the size of the area which is either exposed to (associated with a positive value for ΔC_p) or removed from (negative value for ΔC_p) the aqueous environment during the binding event (Connelly & Thomson, 1992; Murphy & Freire, 1992; Perozzo et al, 2004; Spolar & Record, 1994). ΔC_p and ΔASA are connected by the empirically determined relation $\Delta C_p = \Delta c_{ap} \cdot \Delta ASA_{ap} + \Delta c_p \cdot \Delta ASA_p$ where Δc_{ap} (0.45) and Δc_p (-0.26) are the area coefficients in $cal \cdot K^{-1} \cdot (mol \cdot Å^2)^{-1}$ for the contributions of apolar or polar side chains to ΔASA, respectively (Murphy et al, 1992; Murphy & Freire, 1992; Perozzo et al, 2004). As previously described (Kuhle & Ficner, 2014), we use two values for the area coefficients to estimate the change in surface area upon ligand binding: $\Delta c_{max} = 0.24$ ($= 0.7 \cdot \Delta c_{ap} + 0.3 \cdot \Delta c_p$) as the upper limit case and $\Delta c_{min} = 0.45$ as the lower limit case in which all involved residues are apolar.

Steady-state fluorescence measurements

Fluorescence measurements were carried out on a Fluoromax-3 spectrophotometer (Jobin Yvon Inc.) using a 1 ml quartz cuvette with magnet stirrer. Titrations of eIF5B constructs with mant-GTP were performed at 20 °C in buffer containing 25 mM Tris/HCl (pH 7.5), 200 mM KCl, 3 mM $MgCl_2$ and 2 mM DTT. Binding of mant-GTP to eIF5B constructs was monitored by tryptophan Foerster Resonance Energy Transfer (FRET) using an excitation wavelength of 290 nm and an emission wavelength of 440 nm. In order to estimate the equilibrium dissociation constant (K_d) between eIF5B constructs and mant-GTP, 2 μM of the protein was titrated with increasing amounts of the mant-nucleotide (dilution was less than 1%). The resulting signal was corrected for the contribution of unbound nucleotide by titrating mant-GTP into buffer and subsequently subtracting these values from the signals obtained with protein. The titration data were analyzed using a quadratic binding model:

$$F = \frac{F_0 + \Delta F_{max}\left((X+Y+K_d) - \sqrt{(X+Y+K_d)^2 - 4XY}\right)}{2X}$$

where F is the fluorescence signal of the mant-nucleotide in the presence of eIF5B, F_0 is the initial fluorescence signal, ΔF_{max} is the maximum fluorescence signal, X is the total concentration of eIF5B, Y is the total concentration of the added mant-nucleotide and K_d is the equilibrium dissociation constant.

Supplementary References

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