Supplementary Methods

The structural basis of blebbistatin inhibition and specificity for myosin II

John S. Allingham, Robert Smith, and Ivan Rayment

Protein purification. The truncated *Dictyostelium* myosin II (S1dC) gene containing residues Asp2-Asn762 was cloned into a recombinant version of the pDXA vector (pDTEV2), which inserts a 6-His tag followed by an rTEV protease recognition site at the N-terminus of myosin. This construct was expressed and purified from *Dictyostelium discoideum* as previously described with the following modifications[1]. Approximately 50 g of frozen cells were thawed in 200 mL of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.2 mM EGTA, 20% ethylene glycol, 0.5 mM TCEP, 5 μg/ml leupeptin, 1 mM PMSF, 0.1 mM TLCK, 1 Complete EDTA-free protease inhibitor tablet (Roche) at 4°C. Lysis was initiated by the addition of 100 mL of the above buffer supplemented with 1% triton X-100, 15 ug/ml RNase A, and 100 Units of alkaline phosphatase with continued incubation at 4°C for 1 hour. Cytoskeletal components were collected by centrifugation at 230 000g for 1 h. Pellets were resuspended in 100 ml of 50 mM HEPES (pH 7.3), 30 mM potassium acetate, 10 mM $MgSO_4$, 20% ethylene glycol, 150 mM NaCl, 7 mM β-mercaptoethanol, 5 μg/ml leupeptin, 1 mM PMSF, and 0.1 mM TLCK, and homogenized with a large glass tissue homogenizer at 4°C. The sample was repelleted at 230 000g for 45 min. Myosin was extracted from the pellets by homogenization into 80 mL of the above buffer supplemented with 10 mM ATP. Actin and other cytoskeletal components were removed by centrifugation at 250 000g for 90 min. The supernatant was applied to a 2 ml Ni-NTA agarose column at 1 ml/min that had been equilibrated with the above buffer. The column was washed with 15 volumes of 50 mM Hepes (pH 7.3), 300 mM potassium acetate, and 3 mM

benzamidine, followed by 10 volumes of 50 mM Hepes (pH 7.3), 30 mM potassium acetate, and 3 mM benzamidine. Myosin was eluted with a linear gradient of 20 to 500 mM imidazole (pH 7.3), in 3 mM benzamidine at a flow rate of 1 ml/min. Myosin fractions were pooled and dialyzed for 16 h in 1 L of 10 mM Hepes (pH 7.4), 50 mM NaCl, 0.2 mM EDTA, 0.5 mM TCEP, 0.2 mM NaN_3, and 2 Complete EDTA-free protease inhibitor tablets. The 6-His tag was removed by rTEV protease digestion for 16 h at 4°C during a second round of dialysis using the previous buffer without the addition of protease inhibitor. Myosin was purified from the His-tag by passage over a Ni-NTA agarose column equilibrated with 10 mM Hepes (pH 7.4), 50 mM NaCl, 10 mM imidazole, and 7 mM β-mercaptoethanol. Myosin fractions were pooled and dialyzed as previously described. The protein was concentrated to 10-12 mg/ml in a Centriprep-50 microfiltration device, frozen as 30 μl droplets in liquid nitrogen, and stored at -80°C.

Crystallization. Prior to crystallization, the blebbistatin-bound MgADP.Vi-myosin complex was formed by the addition of blebbistatin (Toronto Research Chemicals) at a final concentration of 0.5 mM followed by a 1/10th volume of a 10X MgADP.Vi stock solution containing 10 mM $MgCl_2$, 20 mM ADP, and 30 mM sodium vanadate[2]. This complex was incubated on ice in the dark for 1 h. Crystals were grown in the dark at 4°C using the hanging-drop method by mixing 5 μl of protein with an equal volume of well solution containing 100 mM MOPS (pH 7.0), 250 mM $MgCl_2$, 11% PEG 8000, 1 mM TCEP, and 2 mM Thymol. The protein/precipitant solutions were streak-seeded from preliminary crystals and grew to maximum dimensions of $0.3\times 0.05\times 0.05$ mm within 2 to 3 weeks.

Data Collection. Crystals were transferred from the original drop to a synthetic mother liquor containing 100 mM MOPS (pH 7.0), 250 mM $MgCl_2$, 2 mM thymol, 12% PEG 8000, 1X MgADP.Vi trapping solution, and 0.5 mM blebbistatin. Prior to freezing, the crystals were

gradually transferred from the synthetic mother liquor to a cryoprotectant solution of 100 mM MOPS (pH 7.0), 300 mM $MgCl_2$, 16% PEG 8000, 2 mM thymol, 1X MgADP.Vi trapping solution, 0.5 mM blebbistatin, and 25% ethylene glycol in 25%, 50 %, 75%, and then 100% increments. The crystal was then picked up in a nylon loop and frozen in a stream of nitrogen gas. X-ray data were collected on a MAR-165 detector at COM-CAT 32-ID at the Advanced Photon Source in Argonne, Il with a wavelength of 0.708 Å. An oscillation range of 0.5° was used, and 280 frames were recorded with a crystal to detector distance of 150 mm. Diffraction data were integrated and scaled with the program HKL2000[3].

Structure determination and refinement. The structure was solved by molecular replacement using the MgADP.Vi-S1dC structure (PDB accession code 1VOM) as the starting model. The structure was refined with Refmac5 and water molecules were added to the coordinate set with ARP/wARP with subsequent manual verification[4,5]. Blebbistatin was built into the electron density by generating a model structure of the inhibitor using the Dundee PRODRG2 server[6], followed by subsequent manual atom position refinement in TURBO[7]. Data collection and refinement statistics are shown in Table 1. Ramachandran statistics show the fraction of residues the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran diagram to be 92.7, 7.1, 0.2, and 0, respectively[8].

Structure analysis and figure preparation. Structure alignments were performed with Superpose and UW_Align[9]. Legend-protein contacts were obtained through the LPC suite (http://bip.weizmann.ac.il/oca-bin/lpccsu/). The total molecular surface area buried at the complex interface was determined with CNS version 1.1. Figures were prepared using MacPymol[10].

Actin-activated MgATPase assay. The actin-activated MgATPase activity was measured with an NADH-coupled assay at 25°C in a Beckman DU640B spectrophotometer at 340 nm[11]. All ATPase reactions contained 50 nM *Dictyostelium* myosin II in 4 mM MOPS (pH 7.1), 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM NaN_3, 40 units/ml lactate dehydrogenase, 200 units/ml pyruvate kinase, 1 mM phosphoenolpyruvate, and 200 µM NADH. Actin concentrations varied from 0 to 20 µM. Reactions were initiated by the addition of 100 µM ATP. Blebbistatin was added from stocks dissolved in DMSO and the DMSO concentration was maintained at 1%.

References:

1. Fisher, A.J. et al. *Biochemistry* **34**, 8960-8972 (1995).
2. Smith, C.A. & Rayment, I. *Biochemistry* **35**, 5404-5417 (1996).
3. Otwinowski, Z. & Minor, W. *Methods in Enzymology* **276**, 307-326 (1997).
4. Murshudov, G.N., Vagin, A.A. & Dodson, E.J. *Acta Crystallogr D Biol Crystallogr* **53**, 240-255 (1997).
5. Perrakis, A., Harkiolaki, M., Wilson, K.S. & Lamzin, V.S. *Acta Crystallogr D Biol Crystallogr* **57**, 1445-50. (2001).
6. Schuttelkopf, A.W. & van Aalten, D.M. *Acta Crystallogr D Biol Crystallogr* **60**, 1355-63 (2004).
7. Roussel, A. & Cambillau, C. *Silicon Graphics Geometry Partners Directory*, Vol. 86 (Silicon Graphics, Mountain View, California, 1991).
8. Laskowski, R.A., MacArthur, M.W., Moss, D.S. & Thornton, J.M. *Journal of Applied Crystallography* **26**, 283-291 (1993).
9. Cohen, G.H. *Journal of applied crystallography* **30**, 1160-1161 (1997).
10. DeLano, W.L. *The PyMOL Molecular Graphics System*, http://www.pymol.org, (2002).
11. Wang, F. et al. *J Biol Chem* **278**, 27439-48 (2003).