Materials and Methods (III)

Acetone Powder Preparation for G-actin Purification

All steps were carried out at 4°C in the cold room. Furthermore, all materials and buffers involved in the purification were incubated over night in the cold room before further use.

Materials

Small scissors
Meet grinder
Cheese cloth (sterilized)
Glass rods
4L Buckets
1000 g very fresh skinless, boneless chicken breast

Extraction Buffer (I)

0.1 M KCl
0.15 M Potassium phosphate, pH 6.5
Ice cold

Extraction Buffer (II)

0.05 M $NaHCO_3$
Ice cold

Extraction Buffer (III)

1 mM EDTA, pH 7.0
Ice cold

G-Actin Extraction

All fat or remaining blood vessels were removed from the meat. The meat was cut into smaller pieces and finely ground. All connecting tissues, that were building up during the grinding procedure were removed periodically.

First Extraction

The mince was stirred for 10 minutes in 1 L ice cold extraction buffer (I). A sterile cheese cloth was used to filter the extract.

Second Extraction

The mince was further stirred in 2 L ice cold extraction buffer (II) for 5 minutes. Care was taken not to exceed the extraction time in this buffer as it reduces the actin yield. The extract was filtered using a sterile cheese cloth.

Third Extraction

The mince was stirred 10 minutes in 1 L ice cold extraction buffer (III) and filtered using a sterile cheese cloth.

Fourth Extraction

The mince was extracted twice in 1 L ice cold water for 5 minutes by constant stirring and filtered using a sterile cheese cloth.

Fifth Extraction

The final five extractions were each in 1 L ice cold acetone for 10 minutes by carefully stirring with a glass rod. The extractions were done at room temperature in a fume hood but the acetone was kept ice cold at all times. Each extraction was filtered using a sterile cheese cloth.

The final extract was spread in a large tray formed with aluminum foil. The tray was covered with a clean cheese cloth, taking care that the cloth did not touch the mince. The mince was air dried in a hood over night. Using gloves the dried acetone powder was filled into 50 ml Falcon tubes and stored at - 80°C. The acetone powder is stable for several months.

G-Actin Purification from Chicken Muscle Acetone Powder

11 g of acetone powder was stirred vigorously in 220 ml of G-buffer for 30 minutes at room temperature. The supernatant was carefully poured off using a glass rod to minimize powder loss.

The first extraction step was repeated at 4°C in the cold room for 5 minutes and the supernatant was poured off as described above. The supernatants were combined and spun in 40 ml Oakridge tubes for 30 minutes at 19000 rpm in a JA-20 Beckman centrifuge. The supernatant was decanted into a cylinder to measure its volume. 5 ml (3M) KCl (50 mM final), 0.6 ml (1M) $MgCl_2$ (2 mM final), 3 ml (0.1 M) ATP (1 mM final) were added to 300 ml of supernatant. The mixture was incubated 60 minutes at room temperature by very slow stirring.

The KCl concentration was raised to 0.6 M to purify the actin from any actin binding protein, particularly from tropomyosin. The mixture was stirred for another 15 minutes and spun at 42000 rpm for 60 minutes in Ti45 tubes. The supernatant was decanted off and 1 ml G-buffer was added per tube. The pellets were combined and homogenized in the smallest volume possible. The homogenized pellets were dialyzed into 1 L G-buffer over night with two changes of buffer. The dissolved pellets were spun at 95000 rpm for 20 minutes. After the concentration of the G-actin in the supernatant was determined, the protein was aliquoted, flash frozen in liquid nitrogen and stored at -80°C.
The determination of the G-actin concentration:
[G-actin (mg/ml)] = (OD290/0.62) ^{x} dilution factor

G-Buffer
2 mM Tris pH 8
0.2 mM ATP
0.2 mM $CaCl_2$
0.1 mM βME

Insertion of the CCXXCC-motif into the wild type S1 Gene

The available over-expression pTIKL OE plasmid was used to sub-clone the wild type *Dictyostelium* myosin II S1 gene into a commercially available pSk^{+} plasmid to facilitate the QuikChange® mutagenesis using Nco I and Xba I restriction sites. After ensuring the CCXXCC insertion via custom DNA

sequencing the heavy chain was cloned back into the pTIKL OE plasmid for protein expression in AX3-Orf$^+$ cells.

Restriction Digest Reactions of the pTIKL OE and pSk+ Plasmids

3 µl Plasmid DNA

1 µl Xba I (20000 U/ml)

1 µl Nco I (10000 U/ml)

1 µl BSA (10 mg/ml)

1 µl (10X) NEB 2 buffer

3 µl dd H_2O

10 µl Final volume

Ligase reaction to create the psK$^+$-*wt*-S1 plasmid

Uncut Vector Control	Vector Religation Control	Ligase Reaction	
1.5 µl	1.5 µl	1.5 µl	10X Ligase Buffer (NEB)
0 µl	0 µl	9 µl	NcoI / Xba I digested psK$^+$ plasmid
3 µl	3 µl	3 µl	NcoI / Xba I digested pTIKL OE plasmid
0 µl	1 µl	1 µl	T4 DNA Ligase (1U/µl) (NEB)
10.5 µl	9 µl	0 µl	10 mM Tris pH 8.5

The ligase reactions were transformed into chemically competent *E. coli* DH5-α cells and the plasmids were purified via QIAprep® Spin Miniprep Kit as described previously.

Site directed QuikChange® Mutagenesis and Primer Design

The wild type S1 heavy chain gene in the psK$^+$ plasmid was mutagenized via QuikChange® site-directed mutagenesis kit. Two mutant constructs (M-724 and M-751) of the *Dictyostelium* myosin II S1 were created by introducing the CCXXCC-motif into different sites in the S1 heavy chain gene. Both of the mutagenic primer pairs contained the desired mutations (shown in orange in

the forward primers) and annealed to the same sequence on opposite strands of the plasmid. The desired mutation should have 10 - 15 flanking bases of complementary sequence on each side of the primer. The GC content should be greater than 40% and should terminate in one or more G or C bases to ensure efficient annealing to the template. Furthermore, primers should ideally have a melting temperature (T_m) greater than or equal to 78°C.

Following equation was used to determine the melting temperature:

$T_m = 81.5 + 0.41(\%GC) - 675/N - \%$ mismatch (N= primer length in bases)

T_m for the M-724 mutagenesis primers was 72°C.

T_m for the M-751 mutagenesis primers was 77.5°C.

Forward Primer for M-724 (Fwd 09)

5'- CACAAAAAGCCACCTGTTGTGTTCTCTGCTGTCTTAACATTGATCC-3'

Reverse Primer for M-724 (Rev 09)

5'-GGATCAATGTTAAGACAGCAGAGAACACAACAGGTGGCTTTTTGTG-3'

Forward Primer for M-751(Fwd 10)

5'-CCGTGCCGGTCAATGCTGTCGTATTTGCTGCGCTCGTGAACAACG-3'

Reverse Primer for M-751(Rev10)

5'-CGTTGTTCACGAGCGCAGCAAATACGACAGCATTGACCGGCACGG-3'

The PCR products were transformed into chemically competent *E. coli* DH5-α cells and the plasmids were purified via QIAprep® Spin Miniprep Kit as described previously. The mutagenized *Dictyostelium* myosin II S1 gene was cloned back into the pTIKL OE plasmid for protein expression.

PCR Reaction to Introduce the CCXXCC-Motif

5 µl (10X) Reaction buffer

1 µl (10 mM) dNTP mix

125 ng Forward primer

125 ng Reverse primer

5 ng - 70 ng Template plasmid

1 µl (2.5U/µl) *PfuTurbo*® DNA Polymerase (Stratagene®)

50 µl Final volume

QuikChange® PCR Method

Step	Time	Temperature	Cycles
Initial Denaturation	30 s	95°C	1X
Denaturation	30 s	95°C	18X
Annealing	1 min	55°C	
Extension	12 min	68°C	
Final Extension	5 min	72°C	1X

Restriction Digest Reactions of the pTIKL OE and pSk+ Plasmids

3 µl Plasmid DNA

1 µl Xba I (20000 U/ml)

1 µl Nco I (10000 U/ml)

1 µl BSA (10 mg/ml)

1 µl (10X) NEB 2 buffer

3 µl dd H_2O

Ligase Reaction to create the pTIKL OE-*CCXXCC*-S1 Plasmid

Uncut Vector Control	Vector Religation Control	Ligase Reaction	
1.5 µl	1.5 µl	1.5 µl	10X Ligase Buffer (NEB)
0 µl	0 µl	9 µl	Nco I / Xba I digested psK^+ vector
3 µl	3 µl	3 µl	Nco I / Xba I digested pTIKL OE vector
0 µl	1 µl	1 µl	T4 DNA Ligase (1U / µl) (NEB)
10.5 µl	9 µl	0 µl	10 mM Tris pH 8.5

The ligase reactions were transformed into chemically competent *E. coli* DH5-α cells and the plasmids were purified as described previously.

Electroporation of Plasmids into AX3-Orf+ Cells

AX3-Orf+ cells were counted using a hemocytometer and centrifuged in a Beckman TJ-6 centrifuge at 7000 rpm for 10 minutes at 4°C in a 15 ml Falcon Tube. The cells were washed in ice cold electrophoresis buffer (E-buffer) via vortex and centrifuged at 7000 rpm for 10 minutes. 5 - 10 µl (~15 µg) of DNA plasmid was pipetted into a 4 mm GenePulser® cuvette (Bio-Rad) on ice. The centrifuged cells were combined and dissolved to 10^7 cells/ml in ice cold E-buffer. 500 µl of the suspended cells were pipetted into each cuvette. The plasmids were transformed at 1.3 kV and 3 µFD capacity using a Bio-Rad GenePulser®. The cuvettes were immediately removed on ice for 5 minutes. 100 µl and 400 µl of the transformed cells were pipetted into a 10 cm Petri dish and supplemented with 10 ml DDHL-5 buffer without additives. After 30 minutes the cells were examined under an inverted microscope. Successfully transformed cells appeared healthy and attached to the dish. Next day the media was replaced with 10 ml DDHL-5 buffer containing 100 U/ml penicillin, 100 µg/ml streptomyosin and 15 µg/ml G418. All media were autoclaved.

100 ml E-Buffer

1.71g Sucrose

1 ml (1M) $NaPO_4$ pH 7.0

Sterile filtered

1L HL-5 pH 6.4

10 g Proteose peptone

5 g Yeast extract

10 g Glucose

0.35 g $Na_2HPO_4*7H_2O$

0.35 g KH_2PO_4

1L (5X) HL-5 pH 6.5

50 g Proteose peptone

13 g Yeast extract

10 g Glucose

0.46 g Na_2HPO_4*12H_2O

0.36 g KH_2PO_4

5L FM Media

0.085 g $NaHCO_3$

5 L unit FM media powder

5 L ddH_2O

500 ml DDHL-5 media

100 U/ml penicillin

100 µg/ml streptomyosin

15 µg/ml G418

5 ml 5X HL-5 pH 6.5

50 ml FM media

445 ml HL-5 pH 6.5

Culturing transformed Dictyostelium Orf+ Cell Lines

Cells were cultured in DDHL-5 media at 22°C either in Petri dishes or in conical flasks on a gyratory shaker at 190 rpm. The cell density was kept between 10^4 - 10^6 cells/ml. For protein expression the transformed cells in a 10 cm Petri dish were quantitatively removed into a 125 ml flask with 25 ml DDHL-5 total volume. For all subsequent inoculations in larger volumes the starting density of the cells was 10^4 cells/ml. Cells were harvested at a cell density of 10^6 cells/ml by centrifugation in a Beckman J-6M swinging bucket centrifuge at 3200 rpm for 10 minutes. Pellets were resuspended in the lysis buffer in a (1:1) volume ratio. The resuspended cells were frozen by pipetting drops into liquid nitrogen and stored at -80°C.

Myosin II S1 Protein Purification

Myosin S1 Purification via $(His)_6$ tag

In a water bath, 200 g cell pellet was thawed in 200 ml lysis buffer in a water bath. To deplete the endogenous ATP in the cells, 25 ml aliquots were removed into JA20 tubes and incubated on ice for one hour. To pellet the actin bound

myosin S1, the lysate was centrifuged at 20000 rpm in a Beckman J2 HS centrifuge for one hour. The pellets were resuspended each in 10 ml extraction buffer and pooled. After adding another 140 ml of extraction buffer the pooled fractions were centrifuged again at 20000 rpm for 30 minutes. Each pellet was resuspended using a glass homogenizer in 10 ml extraction buffer containing 10 mM ATP to release the actin bound myosin S1. The volume was brought up to 100 ml and the extract was centrifuged in a Beckman ultra centrifuge L8-M for one hour at 55000 rpm in a type 60Ti rotor (Beckman). The supernatant was loaded at 2 ml/min onto a Ni-NTA column (FPLC) that was equilibrated with the low salt buffer. The column was washed with 3 column volumes of the low salt buffer followed by 3 column volumes of the high salt buffer to remove unspecifically bound proteins. After the salt wash, the buffers were replaced with imidazole buffers and the column was washed with 3 column volumes of the 20 mM imidazole buffer. The protein was eluted with an imidazole buffer gradient from 20 mM to 500 mM in 15 minutes. The flow rate was 1 ml/min for all wash- and elution steps. The protein containing fractions were pooled and dialyzed into the storage buffer over night or into the zero salt buffer for subsequent ion exchange chromatography (see below). The protein in the storage buffer was concentrated using an Amicon Ultra-4 (Millipore) concentrator (MWCO 30000) and stored at -80°C in appropriate aliquots.

Lysis Buffer

50 mM Tris pH 8.0
2 mM EDTA
1 mM βME
80 µg/ml TPCK
2 µg/ml Pepstatin A
5 µg/ml Leupeptin
50 µg/ml TLCK
0.1 mM PMSF
0.5 µg/ml RNAse

Extraction Buffer

25 mM Hepes pH 7.4

30 mM KOAc

10 mM $MgCl_2$

1 mM βME

80 µg/ml TPCK

2 µg/ml Pepstatin A

5 µg/ml Leupeptin

50 µg/ml TLCK

Low Salt Buffer

50 mM Hepes pH 7.4

30 mM KOAc

3 mM Benzamidine

High Salt Buffer

50 mM Hepes pH 7.4

300 mM KOAc

3 mM Benzamidine

20 mM imidazole Buffer

25 mM Hepes pH 7.4

300 mM NaCl

10 mM $MgCl_2$

1 mM βME

2 mM ATP

20 mM imidazole pH 7.4

500 mM imidazole Buffer

25 mM Hepes pH 7.4

300 mM NaCl

10 mM $MgCl_2$

1 mM βME

2 mM ATP

500 mM imidazole pH 7.4

Storage Buffer

25 mM Hepes pH 7.4

25 mM NaCl

10 mM $MgCl_2$

0.1 mM TCEP

Myosin S1 Purification via Anion Exchange Chromatography

To obtain higher purity, the myosin S1 (wild type and M-751) protein was purified over a MonoQ column (FPLC). The protein containing fractions from the Ni-NTA purification were dialyzed into the no salt buffer over night with two buffer exchanges. The protein was loaded onto a MonoQ column that was equilibrated with no salt buffer. The column was washed with 3 column volumes of the no salt buffer. The protein was eluted with a salt gradient from no salt (0 M NaCl) to high salt buffer (0.5 M NaCl) in 20 minutes. The flow rate was 1 ml/min for all wash and elution steps. The protein containing fractions were pooled and dialyzed into the storage buffer over night with two buffer exchanges.

No salt Buffer

10 mM Tris pH 8

0.25 mM $CaCl_2$

0.1 mM EGTA

0.2 mM βME

High salt Buffer

10 mM Tris pH 8

0.25 mM $CaCl_2$

0.1 mM EGTA

0.2 mM βME

0.5 M NaCl

Storage Buffer

25 mM Hepes pH 7.4

25 mM NaCl

10 mM $MgCl_2$
0.1 mM TCEP

RLC Phosphorylation via MLCK-A Kinase

MLCK-A Protein Expression and Purification

The myosin light chain kinase A (MLCK-A) mutant T166E was expressed using the pET15b expression plasmid to N-terminally $(His)_6$ tag the protein. After the purification, the $(His)_6$ tag was removed by cleavage with thrombin[72]. The plasmid was transformed into the *E. coli* strain BL21. A 6 ml saturated culture from a single colony was used to inoculate a 1 L LB culture complemented with carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The cells were grown at 28°C because higher temperatures led to insoluble protein. The cells were induced with 0.4 mM IPTG at an OD_{600} of 0.45. After 4 hours the culture was centrifuged at 7000 rpm for 10 minutes in a Beckman J-6M swinging bucket centrifuge. The pellet was resuspended in 20 ml lysis buffer and frozen in liquid nitrogen drops. The cells lysed upon thawing. Immediately after thawing the cells, 10 mM $MgCl_2$, 1 mM PMSF, and 5 µg/ml DNAse I were added and the mixture was stirred about 15 minutes at room temperature until the viscosity of the solution decreased. The lysate was centrifuged 15 minutes at 17000 rpm in a Beckman JA20 rotor at 4°C. The supernatant was loaded onto a Ni-NTA column that was previously equilibrated with the 20 mM imidazole buffer. The column was washed with 3 column volumes with 20 mM imidazole buffer. The protein was eluted with an imidazole gradient from 20 mM to 500 mM in 20 minutes. The flow rate was 1 ml/min for all wash and elution steps. Protein containing fractions were identified by SDS PAGE and pooled. To cleave the $(His)_6$ tag, 5 mg Ni-NTA purified MLCK-A protein was dialyzed into the cleavage buffer with freshly prepared 8 U/ml thrombin. For the cleavage reaction, the protein was incubated at 37°C for 50 minutes and quenched with 1 mM PMSF. The protein was loaded onto a Ni-NTA column that was equilibrated with the cleavage buffer to remove the cleaved $(His)_6$ tag. The immediate flow through contained the cleaved MLCK-A protein. 50% glycerol was added to the flow

through for storage purposes and the aliquots were stored at -80°C. After thawing an aliquot, the MLCK-A protein was stored in -20°C for subsequent use.

Lysis Buffer

20 mM Tris pH 7.9

5 mM imidazole

500 mM NaCl

Cleavage Buffer

20 mM Tris pH 8.4

150 mM NaCl

2.5 mM $CaCl_2$

20 mM imidazole buffer

20 mM Tris pH 7.9

5 mM Imidazole

500 mM NaCl

20 mM imidazole

500 mM imidazole buffer

20 mM Tris pH 7.9

5 mM Imidazole

500 mM NaCl

500 mM Imidazole

RLC Kinasing Reaction

To phosphorylate the RLC in *Dictyostelium* myosin II S1, the protein was incubated in the kinasing reaction buffer with the myosin light chain kinase A (MLCK-A) using a (10:1) ratio protein to MLCK-A for one hour on ice. The quantitative phosphorylation of the RLC was ensured on SDS glycerol gels.

Kinasing reaction buffer

10 mM Hepes pH 7.4

6mM $MgCl_2$

5 mM ATP

1 mM DTT

Urea-SDS-Glycerol Mini-Gel Preparation

30 ml 7.5% Separating Gel, pH 8.6

In a small beaker 15.09 g (100%) glycerol was mixed with 5.63 ml of a 40% acrylamide/1.5% bis-acrylamide solution, 2.5 ml (240 mM) Tris-Glycine (pH 8.6) and 9.87 ml ddH_2O. To polymerize the acrylamide, 16 µl TEMED and 100 µl (50 mg/ml) APS were added and stirred thoroughly before pouring the gels. Each individual gel was overlaid with 200 µl water-saturated butanol. Before adding the 3.5% separating gel, water-saturated butanol was poured off and the gels were rinsed thoroughly with ddH_2O.

15 ml 3.5% Separating Gel, pH 6.8

In a small beaker 7.54 g (100%) glycerol was mixed with 1.3 ml of a 40% acrylamide/1.5% bis-acrylamide solution, 3.85 ml (278 mM) Tris-HCl (pH 6.8) and 3.85 ml ddH_2O. To polymerize the acrylamide, 8 µl TEMED and 100 µl (50 mg/ml) APS were added and stirred thoroughly before pouring the gels.

1 L Electrophoresis Buffer

83.3 ml (240 mM) Tris-Glycine (pH 8.6) was added to 1 L ddH_2O.

Sample Preparation

An equal amount of SDS sample buffer was added to the protein sample and boiled at 100°C for 5 minutes in a heat block. An equal amount of urea sample buffer was added before loading the gel. The gels were run at 3 Watts constant power for about 3 hours.

Stock Solutions

0.5 L (240 mM) Tris-Glycine, pH 8.6

14.6 g Tris Base was dissolved in 400 ml ddH_2O and the pH was adjusted to 8.6 using glycine (~10-20 g). Solution was brought to 500 ml with ddH_2O, sterile filtered (0.45 µm) and stored at room temperature.

100 ml 40% acrylamide/1.5% bis-acrylamide

40 g acrylamide and 1.5 g bis-acrylamide powders were dissolved in 50 ml of ddH_2O by heating to 37°C for several minutes. Solution was brought to 100 ml with ddH_2O, sterile filtered (0.45 µm) and stored light protected at room temperature.

100 ml 278 mM Tris-Glycine, pH 6.8

3.36 g Tris Base was dissolved in 80 ml ddH_2O and the pH was adjusted to 6.8 using (6 M) HCl. Solution was brought to 100 ml with ddH_2O, sterile filtered (0.45 µm) and stored at room temperature.

10 ml urea sample buffer

5.45 g urea was dissolved in 4 ml of ddH_2O by heating to 37°C for several minutes. After adding 30 µl (0.5 M) EDTA, 100 µl (1 M) DTT and a pinch of bromophenol blue powder, the solution was brought to 10 ml with ddH_2O.

100 ml SDS sample buffer

0.7 g Tris Base and 2 g SDS powders were dissolved in 60 ml ddH_2O and 10 ml glycerol was added. The pH was adjusted to 6.8 using (6 M) HCl. Before use, 50 µl (14.3 M) βME was added to 950 µl sample buffer.

ATPase assays

All ATPase assays were carried out in 96-well plate format using Spectra Max 340 $_{pc}$ (Molecular Devices). The steady state ATPase activity of 250 nM wild type and mutant S1 enzymes were determined in the ATPase buffer at 5 µM, 10 µM, 20 µM and 40 µM F-actin concentrations. Because of the increasing viscosity at higher concentrations of F-actin, sheering the filaments by pipetting up and down or slightly depolymerizing it with gelsolin using a (1:100) gelsolin to actin ratio has proven crucial. ATPase activity was determined in an enzyme coupled ATPase assay (Figure 25).

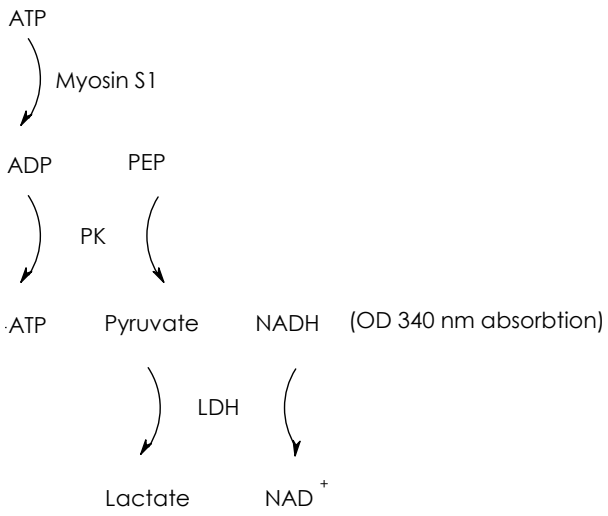

Figure 25: Schematic diagram of the ATP regeneration. Myosin S1 hydrolyzes the ATP and simultaneously the regenerating system of enzymes converts the ADP to ATP. Thus the system maintains a steady state ATPase of the myosin S1protein. For every generated ATP molecule with the PK enzyme one NADH molecule with OD 340 nm is oxidized to NAD^+ leading to a decrease of the absorption at 340 nm.

The output value of the absorption (A) was in [mOD/min] which was then converted to [OD/s]. The extinction coefficient of the NADH (ε_{NADH}) and the diameter (d) of the well allowed the determination of the NADH concentration in the actin activated ATPase assay:

$\varepsilon_{NADH} = 6.22^3 * M^{-1} cm^{-1}$

d= 0.3125 cm

$c_{NADH} = A / \varepsilon_{NADH} * d$

The value of c_{NADH} corresponds to the velocity (v) of the actin activated ATP hydrolysis by a given concentration of actin (A).

Final assay concentrations of the ATP regenerating system

[PK] 0.12 mg/ml

[LD] 0.02 mg/ml

[PEP] 3 mM

[NADH] 0.3 mM

ATPase Buffer

35 mM MOPS pH 7.4

0.1 mM $MgCl_2$

25 mM KCl

2 mM DTT

Probing RLC and HC interaction via Cysteine Footprinting

Cysteine Footprinting via NTCB Labeling

The following protocol can be scaled proportionally depending on the amount of the protein. To alkylate the solvent exposed sulfur residues in the myosin S1, 2 µl of solution I was added to 18 µl (18 µM) protein and incubated for 5 minutes at room temperature. The reaction was quenched by pipetting the reaction mix into 20 µl of solution II. 40 µl (equal volume of the reaction mixture) of solution III was added to label the denatured, therefore solvent exposed, sulfur residues with NTCB. The mixture was incubated 5 minutes at room temperature. 400 µl of 0.05% NaDOC (5X volume of the reaction mixture) was added to the mixture, followed by 48 µl of 50% TCA ($1/10^{th}$ volume of the reaction mixture). It is critical to decrease the GuHCl amount to less than 1 M to ensure the protein precipitation. The reaction was incubated on ice for at least 30 minutes and centrifuged at 14000 rpm for 10 minutes in a benchtop centrifuge. The supernatant was discarded and the pellet was washed with 900 µl ice cold acetone. The pellet was centrifuged at 14000 rpm for 10 minutes in a benchtop centrifuge and the acetone was carefully discarded. The acetone wash was repeated twice to remove as much NTCB as possible to increase the cleavage efficiency. The remaining amounts of NTCB colored the pellet pale yellow upon addition of solution IV. A bright yellow color was indicative of large amounts of NTCB. The pellets were dried in a speed vacuum at room temperature. For the cleavage reaction, the pellets were dissolved in 5 – 7 µl of solution IV and incubated for one hour at room temperature. Cleaved protein bands were analyzed on 15% SDS PAGE.

Solution I

10 µl (1 M) Iodoacetamide

25 µl (2 M) Bicine pH 8.6

65 µl ddH_2O

Solution II

100 µl (10 mg/ml) BSA

3 µl (14.3 M) βME

900 µl ddH_2O

Solution III

150 µl (2 M) Bicine pH 8.6

200 µl (1 M) NTCB

666.7 µl (12 M) GuHCL

Solution IV

780 µl (10M) Urea

20 µl (8M) Ammonia

Cysteine Footprinting Protocol via ICAT labeling

The ICAT solution (1 M) was prepared in 1% TFA and sterile filtered. The protein was denatured in 4 M urea by diluting it in an equal volume of 8 M urea for 10 minutes at room temperature. The denatured protein was incubated 5 minutes with 10 mM iodoacetamide at room temperature and desalted using Micro Bio-Spin P-6 columns (Bio-Rad). The reaction was quenched with 20 mM βME and diluted 10-fold in NaDOC (0.05%). To reduce the disulfide bonds the protein was incubated for 5 minutes with 1 mM DTT. The reaction mixture was desalted using Micro BioSpin P-6 columns (Bio-Rad) to remove the DTT and labeled with 10 mM ICAT for 5 minutes at room temperature. The protein was digested with trypsin in a (1:50) ratio of trypsin to protein over night at 37°C. The presence of the glucose tag in the ICAT label allowed the purification of the digested peptides on a custom made boronate column. The boronate column was prepared in a 3 ml syringe with 500 µl polyacrylamide boronate resin. The resin was equilibrated in 1 ml boron binding buffer. The trypsin digested protein

was applied to the boronate column that was. The peptides were eluted in 250 µl elution buffer and analyzed by MS/MS spectrometry.

50 ml Boron Column Binding Buffer

10 ml (250) mM Hepes pH 9.0

25 ml (1M) NaF

5 ml Acetonitrile

10 ml ddH_2O

Elution Buffer

100 µl (1M) Tris pH 8

500 µl Acetonitrile

1 ml (1M) Sorbitol

3.4 ml ddH_2O

N-(iodoacetyl, p-chlorobenzly)-glucamide (ICAT) synthesis

1 mM (340 mg) of N-*p*-cholorobenzyl-glucamine was weighed into a 50 ml round bottom flask. A freshly made solution of 5 ml Na_2CO_3 (pH 10) was added and stirred until the N-*p*-cholorobenzyl-glucamine was dissolved. 1.2 mM (110 µl) iodoacetylchloride was dissolved in 5 ml dry dioxane in a small beaker. The combined solution of iodoacetylchloride and dioxane was added to the dissolved N-*p*-cholorobenzyl-glucamine and stirred rapidly for 10 minutes. 15 ml chloroform was added to the reaction and removed to a separating funnel and extracted 3 times discarding the bottom layer of chloroform and dioxane. Small amount of NaCl was added to reduce the miscibility if the phases did not separate. The aqueous phases were combined and acidified with approximately 1 ml (6 M) HCl. The pH was reduced below 4 to protonate the acetic acid to force it into the organic phase. 15 ml chloroform was added and the mixture was removed into a separating column. The product was extracted two times and the aqueous phases were combined. To remove the chloroform the aqueous phase was centrifuged in a 15 ml Falcon tube at 4000 rpm in a Beckman J-6M swinging bucket centrifuge for 15 minutes. The supernatant was filtered using a 0.22 micron filter and purified over a reverse phase HPLC using a C-18 column.

HPLC Purification of N-(iodoacetyl, p-chlorobenzly)-glucamide

The C-18 column was washed for 5 minutes at 5 ml/min flow rate with 0.1% TFA (Buffer A). A gradient from 0% to 40% acetonitrile in 0.1% TFA (Buffer B) was used to elute the product. Buffers were degassed with helium for 30 minutes before the HPLC. Following gradient was used for purification:

At 5 min: A = 100%, B 0%
At 30 min: A = 60%, B 40%
At 30.01 min: A = 0%, B 100%
At 35 min: A =0%, B 100%
At 35.01 min: A = 100%, B 0%
At 40 min: A = 100%, B 0%
At 40.01 min: A = 100%, B 0%

The flow rate was at 2.5 ml/min for all steps. The fractions containing the main elusion peak were pooled and the solvent was removed by vacuum centrifugation. To remove the remaining solvent, the product was dried in a freeze dryer (Labconco) over night. The yield was 75%.

K339FlAsH tagged Kinesin Protein Expression and Purification

A (WEAAARECCRECCARA)-tagged kinesin protein was expressed as a positive control for the M-751 and M-724 FlAsH labeling in BL21-Gold competent cells and batch purified using FlAsH-tagged resin. The plasmid encoding the K339FlAsH protein and the FlAsH-tagged resin were kind gifts from Roger Cooke (UCSF).

K339FlAsH protein expression and purification

The plasmid encoding the K339FlAsH protein was transformed into BL21-Gold competent cells as described previously for the transformation of the DH5-α cells. Two 4 L flasks with 1 L TPM media were inoculated each with a 3 ml over night culture grown from a single colony. The cells were grown to OD_{600} = 0.9 and cooled down to 4°C and induced each with 0.9 ml (0.5 M) IPTG. The protein was expressed over night at room temperature. The cells were harvested by spinning at 4000 rpm in a J-6M swinging bucket centrifuge

(Beckman) for 20 minutes and the pellet was resuspended in 50 ml lysis buffer. The cells were lysed using a French Pressure Cell Press (American Instrument Company) and the lysate was centrifuged for 45 minutes in a Beckman JA20 rotor at 4°C. The supernatant was incubated with 1 ml FlAsH-tagged resin by head-over-head shaking for 3 hours at 4°C. Lastly, the protein was incubated for 10 minutes with 1 ml lysis buffer containing 10 mM DMPS to elute the protein.

1L TPM Buffer

20 g Bacto tryptone
15 g Bacto yeast extract
8 g NaCl
2 g Na_2HPO_4 (anhydrous)
1 g KH_2HPO_4 (anhydrous)
Autoclaved in 1 L milliQ water
10 ml (20%) glucose (added at RT)
100 µg/ml ampicillin (added at RT)

Lysis Buffer

20 mM Na_3PO_4
250 mM NaCl
2 mM $MgCl_2$
5 mM βME
2 mM AEBSF
2 mM Leupeptin
2 mM Aprotinin

Myosin II S1 Labeling with FlAsH-EDT$_2$

To work with the same protein concentration for different constructs the protein concentrations of the wild type myosin II S1, M-724 and M-751 were adjusted via Bradford Assay before starting the labeling reaction. The labeling reactions were pursued with varying dye to protein ratios under different nucleotide and pH conditions (Table 1).

Table 1: Conditions for the FlAsH dye labeling of M-751 and M-724 mutant proteins.

Nucleotide	pH	Protein : Dye
10 mM MgATP	7.4	5 : 1
2 mM ADP	8.0	1 : 1
2 mM ADP*VO_4	9.0	1 : 9
2 mM AMP*PNP	-	1 : 50

The FlAsH dye was activated by 15 minutes incubation with DMPS in a 1 µl (1 mM) dye to 1 µl (10 mM) DMPS ratio before the labeling reaction. The protein was incubated with the FlAsH dye for one hour at room temperature in the labeling buffer (150 mM NaCl, 50 mM Hepes pH 7.4, 1 mM TCEP).

Nucleotide State dependent Crosslinking of RLC and HC

The protein was pre-incubated in 0.5 mM DTT before the crosslinking reaction for 45 minutes at room temperature. The incubation with DTT was critical for the nucleotide state dependent crosslinking of the myosin II S1 HC and the RLC. To crosslink the RLC and the HC in the wild type myosin II and the mutant M-751 myosin II, a (1:1) ratio of the protein to DMPS was incubated at room temperature in the ATPase buffer. To take time points, the reaction was quenched in 1X SDS sample buffer that was supplemented with 10 mM DTT and boiled for 5 minutes at 100°C. The crosslinked protein bands were separated on a denaturing SDS-polyacrylamid gel and quantified using the program ImageQuant 5.2.

ATPase Buffer

35 mM MOPS pH 7.4

25 mM KCl

1 mM $MgCl_2$

0.5 mM DTT

± 10 mM ATP