Molecular Biosystems

Interaction and dynamics of the MTIP/MyoA complex, a key component of the invasion motor in the malaria parasite *Plasmodium falciparum*

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ELECTRONIC SUPPLEMENTARY INFORMATION (9 Pages)

Experimental data

1. Protein expression and purification

1.1 MTIP expression

30 mL LB media containing 100 μg/mL ampicillin was inoculated with a colony of BL21(DE3) cells containing the pET46EkLIC plasmid (including a His tag on MTIP) to form a starter culture. This was incubated at 37 °C and shaken at 225 rpm overnight. 20 mL of this culture was then added to 1 L new LB media containing 100 μg/mL ampicillin, and this was shaken at 37 °C for 2.5 hrs until optical density (OD_{600}) measurements reached 0.5–0.6. IPTG (0.1 mM) was added to the flask and it was shaken at 225 rpm, 37 °C for a further 3 hrs. The culture was centrifuged (4000 rpm, 4 °C, 15 mins), the supernatant was removed and the cell pellet was frozen at −20 °C overnight.

The cell pellet was defrosted on ice, to which a solution of 10 mL Bugbuster, 5 μl DNAse and 1 Roche EDTA-free protease inhibitor tablet was added. Once at room temperature the solution was spun on a rotary mixer for 10 mins, then left on ice for a further 10 mins. The cells were then centrifuged at 12,000 rpm, 4 °C for 40 mins. The supernatant containing the protein was filtered through a 0.2 μm syringe (supplied by Nalgene) and retained for purification.

1.2 MTIP CTD and MTIP NTD expression

The same protocol was followed for expression of both CTD and NTD MTIP constructs. 50 mL LB media containing 50 μg/ml kanamycin was inoculated with BL21(DE3) cells containing the pET30XaLIC vector (including both His and S-tags on the protein) to form a starter culture which was incubated at 37 °C and shaken at 225 rpm overnight. 2.5 mL of this was added to 1 L LB medium containing 50 μg/mL kanamycin, and this was shaken at 225 rpm, 37 °C for 2 hrs until the OD_{600} reached 0.5–0.6. IPTG (0.1 mM) was added, and the broth was shaken at 18 °C (225 rpm) overnight. The culture was centrifuged (4000 rpm, 4 °C, 15 mins), the LB broth removed, and the cell pellet washed three times with PBS before being flash frozen in liquid nitrogen and stored at –80 °C.

A solution of 10 mL Bugbuster, 5 μL DNAse and one Roche EDTA-free protease inhibitor tablet was made up, added to the cells after they had been defrosted on ice, and the mixture was vortexed. Once at room temperature the solution was placed on a rotary mixer for 15 mins, then placed on ice for 10 mins. It was centrifuged at 15,000 rpm, 4 °C for 30 mins

and the supernatant removed and filtered through a 0.2 μm syringe (supplied by Nalgene) ready for purification.

1.3 ^{15}N MTIP and ^{15}N MTIP[61-204] expression

50 mL LB broth containing 50 μg/ml ampicillin and 25 μg/ml kannamycin was inoculated with BL21(DE3) cells containing either the pQE30 and pRep4 plasmids (including a His tag on MTIP) for MTIP[61-204], or the pET46EkLIC plasmid for full-length MTIP. This was shaken (225 rpm) at 37 °C overnight, then 24 ml added to each of 2 x 1 L minimal media containing 50 μg/ml ampicillin and 25 μg/ml kannamycin. These were shaken at 225 rpm for 3 hrs until OD_{600} reached ~ 0.5, and they were then induced with 0.1 mM IPTG. The cells were allowed to shake (225 rpm) at 37 °C for 3 hours before the culture was centrifuged (4000 rpm, 4 °C, 15 mins), the minimal media removed, and the cells frozen at -20 °C overnight. A solution of 10 mL Bugbuster, 5 μL DNAse and 1 Roche EDTA-free protease inhibitor tablet was made and added to the mixture, which was allowed to stand on ice for 10 mins to defrost. The solution was spun on a rotary mixer for 15 mins, and then placed on ice for 10 mins. It was centrifuged at 12,000 rpm, 4 °C for 30 mins and the supernatant removed and filtered through a 0.2 μm syringe (supplied by Nalgene) ready for purification.

1.4. Protein purification

Purification was achieved by mixing the soluble lysate obtained from the cells with 4 mL Ni-NTA agarose beads (50 % suspension supplied by Qiagen) that had been washed 3 times with wash buffer (50 mM Tris-Cl, 200 mM NaCl, 5 mM imidazole). The protein solution/Ni-NTA beads mixture was put on a rotary mixer and spun slowly at 4 °C for 1 hr. The liquid was filtered off and the beads washed three times with wash buffer. Elution from the beads was achieved by mixing them with 5 mL elution buffer (50 mM Tris-Cl, 200m M NaCl, 500 mM imidazole). An SDS PAGE gel was run to confirm protein purity, and it was then dialysed overnight into storage buffer (50 mM Tris-Cl, 200 mM NaCl) using a Slide-A-Lyzer 3500 MW dialysis cassette in 2 L buffer at 4 °C. Dialysis was then performed twice more using the same conditions for 1 hr each time.

1.5 Protein concentration determination

Protein concentrations and yields were calculated by UV absorption spectroscopy using ε_{280} = 28,800 $M^{-1}cm^{-1}$ for MTIP, ε_{280} = 17,545 $M^{-1}cm^{-1}$ for ^{15}N MTIP[61-204], ε_{280} = 8,605 $M^{-1}cm^{-1}$ for MTIP CTD and ε_{280} = 20,525 $M^{-1}cm^{-1}$ for MTIP NTD.

2. Peptide Synthesis

All peptides were synthesised using standard Fmoc-protected SPPS methods. Briefly, the chosen resin (25 μmol per well) was swelled in DMF for 60 minutes before deprotection of the N-terminal Fmoc protecting group with 20% piperidine (v/v) in DMF for 15 minutes. This was repeated three times. Five equivalents of the incoming *N*-Fmoc protected amino acid were added (as a 0.5 M solution in DMF), and HOBt/HBTU (125 μmol, 250 μL) and DIPEA (125 μmol, 250 μL) used to activate each amino acid. The resin was then washed 3 times with DMF (1 mL), and the cycle of deprotection, DMF wash, amino acid coupling, DMF wash repeated for each amino acid in the peptide sequence. After the final amino acid coupling, the N-terminal Fmoc group was cleaved using 20% piperidine (v/v) in DMF. The protected resin-bound peptides were then removed from the reaction block, and either acetylated or dansylated in a fritted syringe (see individual sections). Each peptide was then washed with 3 × DMF, 3 × dichloromethane, 3 × methanol and 3 × diethyl ether before being covered and dried in a vacuum desiccator overnight. The peptides were deprotected and cleaved from the resin using a deprotection/cleavage mixture of 94.5% TFA, 2.5% water, 2.5% EDT, 1% TIS. 1 mL of this mixture was added to each resin-bound peptide and they were shaken at room temperature for 3 hrs. The liquid was filtered, and the resin washed with 500 μL deprotection mixture. To each peptide solution was added 10 mL cold tert-butyl-methyl-ether (TBME) to give a precipitate. This was centrifuged (4000 rpm, 4 °C, 15 mins) and the solid washed with 10 mL TBME three more times. The peptides were then placed in a vacuum dessicator overnight before being purified by HPLC and freeze-dried. All peptides were stored in water at –20 °C.

2.1. Acetylation of peptides

Following standard SPPS the peptides were each placed in a 5 mL syringe and the resin swelled by addition of 1 mL DMF followed by shaking at room temperature for 1 hr. This was removed by filtration, and 1 mL acetylation mixture (10% acetic anhydride, 20% DIPEA in DMF) added and shaken at room temperature for 1 hr. The reagent mixture was removed by filtration and the peptides washed and deprotected as above. Characterisation data for these peptides is shown in Table S1.

2.2. Dansylation of peptides

Following removal of the peptides to a syringe from the SPPS reaction block, the resin was swelled using 1 mL DMF shaking for 1 hr. This was then removed by filtration and 1 mL

dansylation reagent mixture (1 mL DIPEA (0.5M in DMF), 1 mL DMF, 70 mg dansyl chloride) added and shaken at room temperature for 1 hr. The reagent mixture was removed, the resin rinsed with DMF, and another 1 mL dansylation mixture added and shaken for 1 hr (double coupling). The reagent mixture was removed and the peptides washed and deprotected as above. Stock concentrations were confirmed by UV absorption spectroscopy using ε_{330} = 4300 $M^{-1}cm^{-1}$. Characterisation data for these peptides is shown in Table S1.

Peptide	Rt (mins)	MALDI-TOF peaks
MyoA[791-818]	19.9	3401 (M+1)
MyoA[791-816]	18.4	3200 (M+1)
MyoA[795-818]	17.8	2844 (M+1)
MyoA[799-818]	18.4	2373 (M+1)
MyoA[800-818]	18.6	2244 (M+1)
MyoA[801-818]	18.9	2130 (M+1)
MyoA[802-818]	18.7	2017 (M+1)
MyoA[803-818]	18.3	1921 (M+1)
MyoA[799-817]	26.0*	2243 (M+1)
MyoA[799-816]	26.3*	2174 (M+1)
MyoA[799-815]	25.4*	2073 (M+1)
MyoA[799-814]	18.3	1943 (M+1)
MyoA[799-813]	18.2	1815 (M+1)
MyoA[781-801]	18.8	2523 (M+1)
MyoA[791-818]P802A	18.5	3374 (M+1)
MyoA[799-818]K813A	25.7*	2287 (M+1)
MyoA[803-818]R806A	18.7	1836 (M+1)
SPMyoA[806-818]G	21*	1848 (M+1)
ranMyoA[803-818]	18.5	1921 (M+1)
d-GMyoA[803-818]	26.8*	2170 (M+1)
d-GMyoA[799-818]	27.5*	2374 (M+1)
d-GMyoA[791-818]	17.9	3648 (M+1)
d-GMyoA[781-818]	19.4	4671 (M+1)
d-GMyoA[781-801]	19.3	2714 (M+1)

Table S1: Characterisation data for all peptides synthesised. * Rt measured using 2-98% H_2O/methanol over 40 minutes. All other Rt values measured using 2-98% H_2O/acetonitrile over 40 minutes.

3. Dansyl intensity binding assay (cuvette)

3 mL of 1 μM d-peptide in assay buffer (25 mM Tris-Cl, 100 mM NaCl, 1 mM EGTA, 1 mM DTT, pH 8.0) was added to a cuvette and the fluorescence emission spectrum taken between 400-600 nm, and a single read taken at 525 nm (excitation = 330 nm). MTIP (250 μM) was

added 2 µL at a time and thoroughly mixed before each reading. Fluorescence readings were taken after each addition at room temperature. 24 µL MTIP (250 µM) was added in total to give a final MTIP concentration of 2 µM.

4. Dansyl intensity inhibition assay (plate)

Assays were carried out in triplicate in the same 96 well microplate, with four separate inhibitors able to be tested at the same time in one plate. A multi-channel pipette was used to add 150 µL assay buffer into wells B-G in rows 12, 9, 6 and 3. 300 µL of Inhibitor 1 was placed in well A12, Inhibitor 2 in A9, Inhibitor 3 in A6 and Inhibitor 4 in A3. Doubling dilutions using 150 µL of each of these inhibitors was carried out between wells A-G to give a concentration range between 1-64 µM. The multi-channel pipette was used to transfer 50 µL from row 12 into rows 10 and 11, 50 µL from row 9 into rows 8 and 7, 50 µL from row 6 into rows 5 and 4, and 50 µL from row 3 into rows 2 and 1 to set up triplicate experiments. 50 µL assay buffer was added to wells 1-12 of column H which was to be used for controls.

7000 µL d-GMyoA[791-818] (2.6 µM) was mixed with 7000 µL MTIP (3.2 µM) and 500 µL d-GMyoA[791-818] (2.6 µM) was mixed with 500 µL assay buffer, and both of these solutions were incubated at 37 °C for 10 mins along with the plate containing the various concentrations of inhibitor. 150 µL of the MTIP (1.6 µM)/d-GMyoA[791-818] (1.3 µM) complex was then added to wells A-G of rows 1-12 to give wells containing the preformed complex with various concentrations of inhibitor. 150 µL was also added to wells H1-H4 to be used as controls. The d-GMyoA[791-818] (1.3 µM) mixed with assay buffer was added (150 µL) to wells H5-H8, and 150 uL buffer was added to H9-H12 to complete the control lane. The plate was incubated at 37 °C for 30 mins before the fluorescence was scanned between 400-600 nm (excitation 330 nm) and single point readings taken at emission 525 nm.

5. ^{15}N SOFAST-HMQC NMR

500 µL ^{15}N MTIP[61-204] (300 µM) was placed in an NMR tube and the HMQC spectra recorded at 600 MHz at 30 °C. Peptide (15 mM) was added in 3 µL additions and the spectra recorded after each addition. 30 µL was added in total to give a final peptide concentration of 900 µM.

6. Live parasite invasion assays – Fluorescence Activated Cell Sorting method (FACS)

Stock parasite cultures of *P. falciparum* were cultured in 2% haematocrit. To generate a synchronous staged parasite population, the parasites were first Percoll treated (centrifuged onto a 70% Percoll cushion) with the late trophozoite/schizonts (which accumulate at the media/Percoll interface) re-cultured for 3-5 hrs, taking smears to assay for merozoites release. Subsequently cultures were Sorbatol treated to remove any remaining schizonts and generate a parasite culture staged within 3-5 hrs of invasion. The synchronized culture was grown overnight so the parasites matured to trophozoite's.

To setup the FACS assay cultures, parasitemia were first gauged using the FACS. In brief, 500µl hydroethidine (HE) fluorescence stain (50 µg/ml in PBS) was placed into a FACS tube to which 50 µl of the culture was added and the tube incubated at 37 °C for 20 minutes. Samples were then removed and placed on ice in the dark for up to 2 hrs. HE labelling was then detected using the FL2 detector (585/42 nm) and parasitemia calculated as a percentage. The FACS count for parasitemia was then used to setup an appropriately diluted culture, typically 1% parasitemia in 2% haematocrit for a one cycle assay. For each assay a 90µl culture is placed into a well of a 96 well plate, and 10 µL inhibitor added at various concentrations to give a final assay volume of 100 µl. The inhibitor stock was diluted using a 10 x stock of DMSO media to give final inhibitor concentrations of 250 µM, 500 µM and 1 mM, all in media containing 0.5% DMSO. All assays were carried out in triplicate.

Plates were incubated at 37°C for approx 44-48hrs i.e. through one invasion cycle to the next trophozoite stage prior to reading. Parasitemia was then calculated using the FACS.

This FACS assay is based on that described by Bergmann-Leither *et al* **75** (3) 2006 *The American Society of Tropical Medicine and Hygiene.*

Supplemental Figures

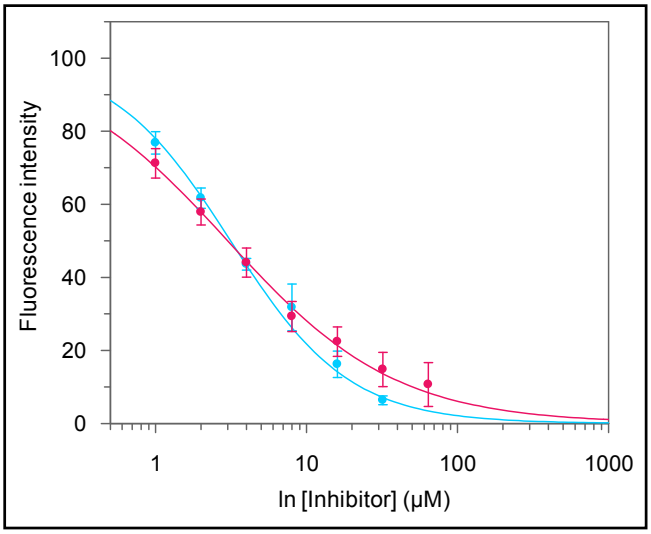

Figure S1: Example of IC_{50} curves for MyoA[791-818] (pink) and MyoA[799-816] (cyan) against MTIP (1.2 μM)/d-GMyoA[791-818] (1 μM). Data was obtained as described in Section 4.

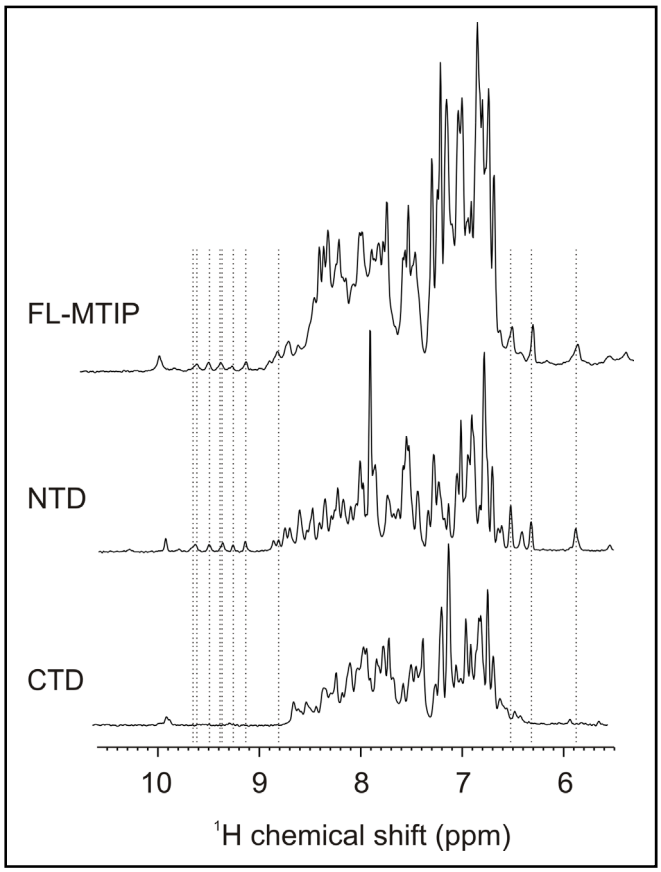

Figure S2: Amide and aromatic region of the proton spectra for full length MTIP (residues 1-204), NTD (1-140) and CTD (141-204). As shown by the dotted lines, most dispersed resonances in full length MTIP correspond to the NTD region.

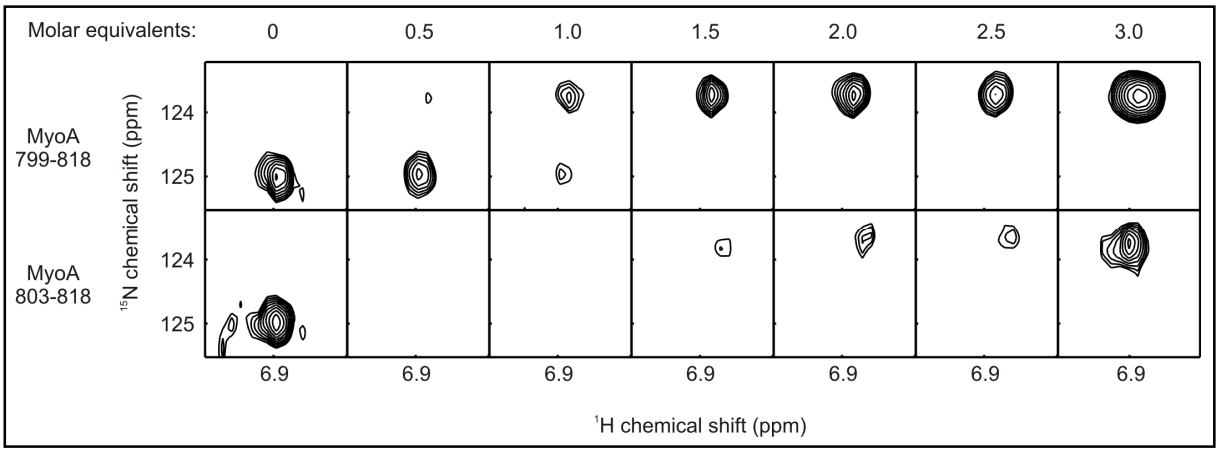

Figure S3: Effect of peptides MyoA[799-818] and MyoA[803-818] on a selected resonance in the ^{15}N SOFAST-HMQC spectra of MTIP[61-204]. Unlike MyoA[799-818], MyoA[803-818] requires larger than equimolar amounts to form an observable complex with MTIP[61-204]. Data was obtained as in Section 5.

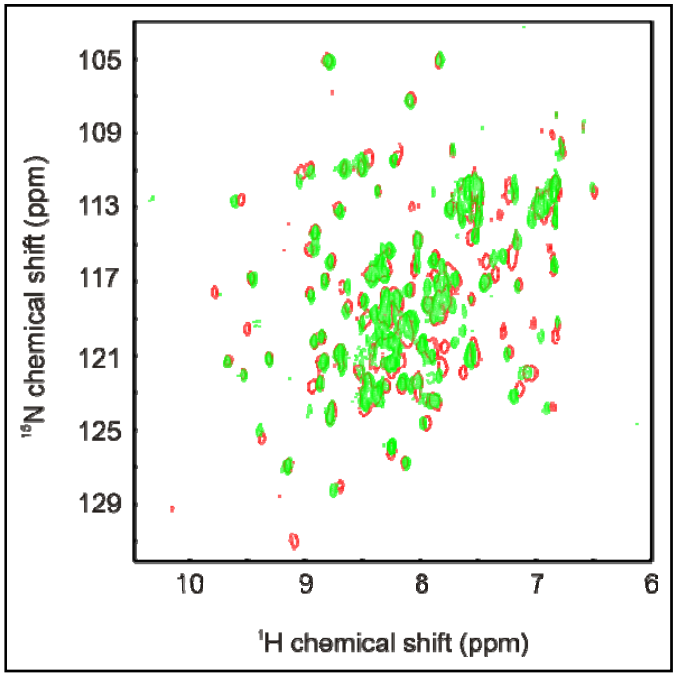

Figure S4: ^{15}N SOFAST-HMQC NMR spectra of MTIP[61-204]/MyoA[803-818] (green) and MTIP[61-204]/d-GMyoA[803-818] (red). The experiment was carried out as in Section 5.

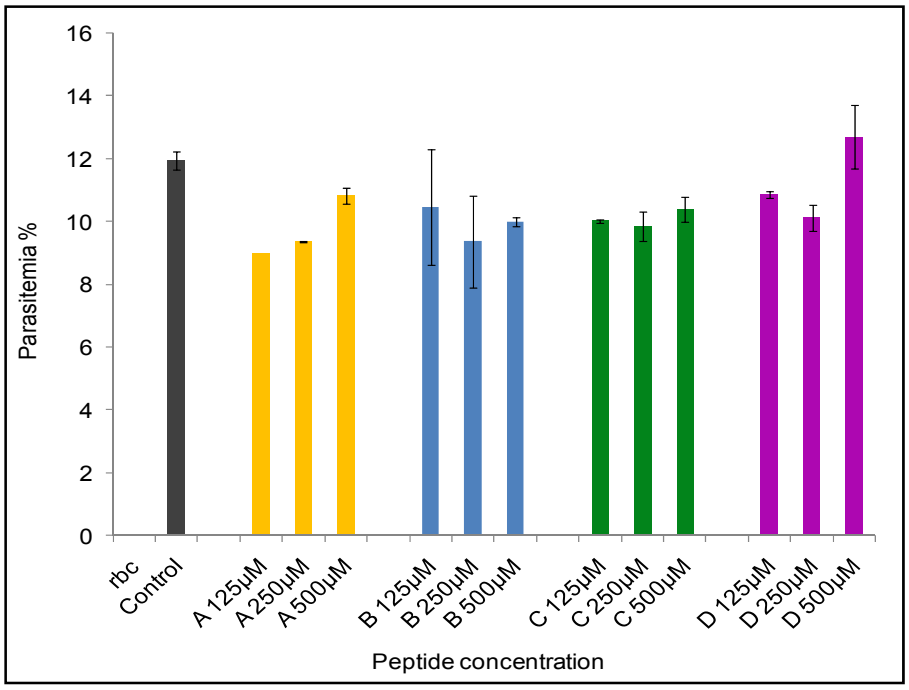

Figure S5: FACS data for MyoA-mimic peptides A) MyoA[791-818] B) MyoA[799-818], C) MyoA[803-818], D) MyoA[803-818]R806A. The data was collected as described in Section 6.