Evidence from NMR interaction studies challenges the hypothesis of direct lipid transfer from L-FABP to malaria sporozoite protein UIS3

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Abstract: UIS3 is a malaria parasite protein essential for liver stage development of *Plasmodium* species, presumably localized to the membrane of the parasitophorous vacuole formed in infected cells. It has been recently proposed that the soluble domain of UIS3 interacts with the host liver fatty acid binding protein (L-FABP), providing the parasite with a pathway for importing exogenous lipids required for its rapid growth. This finding may suggest novel strategies for arresting parasite development. In this study, we have investigated the interaction between human L-FABP and the soluble domain of *Plasmodium falciparum* UIS3 by NMR spectroscopy. The amino acid residue-specific analysis of ^1H , ^{15}N -2D NMR spectra excluded the occurrence of a direct interaction between L-FABP (in its unbound and oleate-loaded forms) and *Pf*-UIS3. Furthermore, the spectrum of *Pf*-UIS3 was unchanged when oleate or phospholipids were added. The present investigation entails a reformulation of the current model of host-pathogen lipid transfer, possibly redirecting research for early intervention against malaria.

Keywords: UIS3; fatty acid binding protein; NMR spectroscopy; protein–protein interaction; malaria; lipid transfer

Introduction

Malaria is a devastating resurgent disease still affecting millions of people worldwide.¹ Infection is initiated when *Plasmodium* sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito. Sporozoites deposited in the skin enter blood vessels and migrate to the liver,

where they infect hepatocytes and begin to develop into merozoites.^{2,3} The latter will be released into the bloodstream, invade erythrocytes and replicate to generate new infectious merozoites.⁴ Malaria-associated pathology originates during the blood stage of *Plasmodium* life cycle while the liver stage is asymptomatic and offers opportunities for early intervention and vaccine development.⁵

Relatively little is known about molecular interactions in either the parasite or the host during the liver stage. The residency and maturation of sporozoites inside host cells require the formation of a membrane-bound vacuole around the parasite.⁶ The lipid and protein composition of the parasitophorous vacuole membrane (PVM) however remains poorly characterized. One model suggests that the bulk of PVM is derived from the host hepatocyte plasma membrane.⁷ Indeed, rapid parasite growth necessitates constant supply of host molecules.

Abbreviations: FABP, fatty acid binding protein; hL-FABP, human liver fatty acid binding protein; HSQC, heteronuclear single quantum coherence; L-FABP, liver fatty acid binding protein; mL-FABP, mouse liver fatty acid binding protein; *Pf*-UIS3, *Plasmodium falciparum* UIS3; *Py*-UIS3, *Plasmodium yoelii* UIS3; PVM, parasitophorous vacuole membrane; UIS, up-regulated in infective sporozoites.

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UIS3 is a small transmembrane protein, presumably localized to the PVM, specifically expressed in sporozoites when they gain infectivity, and essential for early-stage liver development.^{8,9} A central role of UIS3 in fatty acid uptake has been recently proposed.¹⁰ Indeed, a yeast two-hybrid screen based on UIS3 of the rodent malaria parasite *P. yoelii* (Py-UIS3) identified mouse liver fatty acid binding protein (mL-FABP) as an interacting host protein. L-FABP is a cytosolic protein expressed at high levels in hepatocytes and involved in intracellular fatty acid trafficking.^{11,12} L-FABP is able to bind long chain fatty acids with high affinity, as well as a variety of other lipophilic or amphiphilic molecules.^{13–16} The link between L-FABP and UIS3 is enforced by the observation that L-FABP levels in the host cell directly correlate with liver stage growth of *P. berghei*.¹⁰

Rodent malaria parasites are considered excellent models for the description of liver-stage maturation of the human parasite *P. falciparum*⁹ and an involvement of human L-FABP in human malaria development may thus be inferred. Direct interaction between hL-FABP and the soluble C-terminal domain of *Pf*-UIS3 (*Pf*-UIS3(130-229)) was recently shown to occur, based on an ELISA assay.¹⁷ *Pf*-UIS3(130-229) was also crystallized in complex with phosphatidylethanolamine for structure determination,¹⁷ again supporting the involvement of UIS3 in the uptake of lipids for PVM synthesis.

To obtain atomic-level details about fatty acid exchange between host and parasite proteins, the interaction of hL-FABP and *Pf*-UIS3(130-229) was studied by NMR spectroscopy, a technique with recognized great power for the investigation of intermolecular interactions in the field of structure-based drug discovery.¹⁸ We show that ¹⁵N-enriched *Pf*-UIS3(130-229) can be obtained in high yield by heterologous expression and the corresponding heteronuclear 2D spectrum indicates that a well-folded globular protein was obtained. Signals of either [¹⁵N]-hL-FABP or [¹⁵N]-*Pf*-UIS3(130-229) were selectively observed in protein mixtures with unlabeled partners to detect binding-induced perturbations. Fatty acid-loaded hL-FABP and free lipids were also presented to *Pf*-UIS3(130-229) samples. Our spectroscopic characterization rules out any interaction of *Pf*-UIS3(130-229) with hL-FABP and lipid molecules, calling for a redefinition of the current model of FABP-mediated lipid import by human malaria parasites.

Results

Analysis of hL-FABP NMR signals in the presence of *Pf*-UIS3

The predicted full-length structure of UIS3 includes a signal peptide, a short N-terminal region, a transmembrane domain, and a C-terminal domain spanning amino acid residues 83-229.⁹ It is believed that

the C-terminal domain is exposed to the host cytosol, allowing communication between the intravacuolar compartment and the exterior. It was earlier found that recombinantly produced *Pf*-UIS3(83-229) dimerizes in solution, does not crystallize, and undergoes proteolytic cleavage, resulting in a mature monomeric form comprising residues 130-229,¹⁷ *Pf*-UIS3(130-229). The crystal structure of the latter species indicates a compact four-helix globular fold (PDB code: 2VWA). The interaction between hL-FABP and *Pf*-UIS3(130-229) was previously proposed on the basis of an ELISA assay.¹⁷

To analyze the mode of interaction between hL-FABP and the soluble domain of *Pf*-UIS3, NMR interaction experiments were performed. The 130-229 sequence was cloned from *Pf*-UIS3(83-229) to bypass the proteolytic maturation step, resulting in a stable monodisperse protein product, as verified by SDS-PAGE and homonuclear two-dimensional NMR. *Pf*-UIS3(130-229) was mixed at varying molar ratios with ¹⁵N-enriched hL-FABP. Isotopic enrichment allows collecting 2D ¹H,¹⁵N-HSQC NMR spectra that display all amide resonances of the only labeled partner, separated in a proton and a nitrogen frequency dimension (Fig. 1). The residue-specific assignment of cross-peaks in the spectrum of unbound hL-FABP was already available.¹⁶ Because of the inherent sensitivity of amide NMR signals to their proximal chemical environment, even weak molecular interactions are known to be readily detectable from changes in peak position (chemical shift) or intensity. Unexpectedly, step-wise additions up to a three-fold excess of *Pf*-UIS3(130-229) produced only negligible perturbations in the spectrum of hL-FABP (Fig. 1), thereby ruling out any interaction between the two biomolecules in our experimental conditions. Analogous non-significant chemical shift variations were observed in a control experiment with hen egg-white lysozyme, a protein that does not interact with hL-FABP.

Exploring oleate transfer from hL-FABP to *Pf*-UIS3

The structure of hL-FABP is characterized by ten antiparallel beta-strands arranged in a clam shell-like conformation as well as a helix-turn-helix motif surrounding a large interior cavity.¹⁹ The protein is known to be able to accommodate two molecules of long chain fatty acids, determining small changes in overall structure and internal mobility.^{20,21} These changes are reflected by significantly different chemical shift positions of cross-peaks in 2D ¹H,¹⁵N-HSQC NMR spectra of unbound and oleate-bound [¹⁵N]-hL-FABP [Figs. 1 and 2(A)]. The occurrence of incomplete filling of the ligand binding cavity can be readily detected with reference to the appearance of spectra acquired during a ligand-titration experiment (data not shown). It was therefore expected that oleate transfer to UIS3 would result in

Figure 1. Interaction between hL-FABP and *Pf*-UIS3(130-229). The ^1H , ^{15}N -HSQC spectrum of ^{15}N -hL-FABP in the absence (blue) and presence (red) of unenriched *Pf*-UIS3(130-229) is shown. The peak position in the 2D spectrum, in both the nitrogen (y axis) and proton (x axis) dimensions, is indicative of the chemical environment of the corresponding amino acids (over the entire protein structure). Due to the almost identical appearance of the two presented spectra, the spectrum displayed in blue has been offset low-field by 0.05 ppm (^1H) and upfield by 0.3 ppm (^{15}N) for better visualization of peak shapes and intensities. On top of the figure, the structure of the observed hL-FABP is shown in blue and the structure of the undetected *Pf*-UIS3(130-229) in light gray.

significant perturbations of the 2D ^1H , ^{15}N -HSQC spectrum of the fully loaded hL-FABP. The spectrum collected after addition of *Pf*-UIS3(130-229), however, appears almost unchanged compared to that of the isolated hL-FABP:oleate complex [Fig. 2(A)]. Small peak position changes or intensity variations were attributed to the high sensitivity of some solvent-accessible residues to little changes in solution pH, as verified by pH titration experiments. This result indicates that there is a lack of interaction between lipid-bound hL-FABP and *Pf*-UIS3(130-229) and no significant release of bound oleate.

To definitely verify whether partial transfer of oleate molecules to *Pf*-UIS3(130-229) had occurred, we performed a complementary binding assay to observe the signals of the acceptor protein. To this purpose, ^{15}N -*Pf*-UIS3(130-229) was prepared and its structural integrity verified by 2D ^1H , ^{15}N -HSQC

NMR. The spectrum, the first to be shown for a *Plasmodium* UIS protein, displays well-dispersed sharp peaks consistent with a small monomeric globular protein. The number of counted peaks corresponds to the number of detectable amide signals expected on the basis of the primary sequence, indicating that all amino acid residues share the same overall dynamics and no extremely flexible protein segments are present. The addition of an excess of oleate-bound unenriched hL-FABP produced no perturbation in the spectrum of ^{15}N -*Pf*-UIS3(130-229) [Fig. 2(B)]. An additional ^{13}C -observe NMR experiment performed using $^{13}\text{C}_1$ -oleate confirmed the absence of oleate-UIS3 interaction (not shown).

Investigation of lipid binding to *Pf*-UIS3

The observation that hL-FABP is not able to transfer oleate to *Pf*-UIS3(130-229) could be interpreted by a stronger affinity towards oleate of the presumed donor protein compared to that of the acceptor. We therefore explored the lipid binding capability of *Pf*-UIS3(130-229) without hL-FABP mediation. Samples of ^{15}N -*Pf*-UIS3(130-229) were presented with excess oleate, dimyristoyl-phosphatidylcholine, and palmitoyl-oleoyl-phosphatidylcholine. As the observed corresponding spectra (Fig. 3) were unchanged with respect to the free protein, it can be concluded that in our experimental conditions *Pf*-UIS3(130-229) did not bind lipids specifically. It is important to mention that *Pf*-UIS3(130-229) was obtained from the expressed insoluble fraction via a refolding protocol which should in itself exclude the possibility that the obtained protein was bound to lipids of the *E. coli* cells. Moreover, the comparison of NMR spectra of the protein before and after delipidation confirmed that the samples contained pure protein. Finally, the identity of the spectra of refolded *Pf*-UIS3(130-229) and of *Pf*-UIS3(130-229) purified from the soluble fraction demonstrates that the protein was correctly folded.

Discussion

Limited experimental accessibility to *Plasmodium* hepatic stages has so far impeded even a crude characterization of molecular interaction events crucial for parasite maturation. The proposed L-FABP-mediated acquisition of lipids by the malaria parasite UIS3 protein is intriguing, as it would provide novel strategies for arresting the infection. A yeast-two hybrid assay and coimmunoprecipitation experiments indicated interaction between mouse L-FABP and the rodent malaria parasite *Py*-UIS3.¹⁰ A confirmation of the observed binding using biophysical methods is highly demanded in order to exclude the possibility that false positives were detected.²² Nonetheless, the L-FABP:UIS3 interaction was also confirmed for the human system by an ELISA assay,¹⁷ indicating that the C-terminal protein domain, *Pf*-UIS3(130-229), is sufficient to establish the binding.

Figure 2. Interaction between oleate-bound hL-FABP and *Pf*-UIS3(130-229). A: The ^1H , ^{15}N -HSQC spectrum of oleate-bound [^{15}N]-hL-FABP in the absence (blue) and presence (red) of unenriched *Pf*-UIS3(130-229) is shown. B: The ^1H , ^{15}N -HSQC spectrum of [^{15}N]-*Pf*-UIS3(130-229) in the absence (black) and presence (red) of unenriched hL-FABP is shown. The spectra in blue and black have been offset low-field by 0.05 ppm (^1H) and upfield by 0.3 ppm (^{15}N). The structures of the detected (and undetected) proteins are depicted on top of each figure.

It is then highly surprising that no interaction is observed by our NMR study between hL-FABP and *Pf*-UIS3(130-229). It should be mentioned that the proteins in the two investigations are virtually identical, besides the fact that the protein described in the previous study is obtained after proteolytic degradation of *Pf*-UIS3(83-229). The NMR method has the invaluable advantage that binding is tested with the possibility to simultaneously monitor preservation of structural integrity of both putative partners. Thus, it can be hypothesized that the protein products used in biochemical binding assays assume non-natural conformations that expose binding-prone amino acids. Alternatively, the presence of artificial terminal tags may have changed the native binding properties. It also has to be remarked that the NMR binding study was carried out using concentrations which are, at least for hL-FABP, close-to-physiological,²³ and should therefore report on *in-vivo* binding affinities. The absence of chemical shift variations, $\Delta\delta_{\text{HN}}$ (calculated as indicated in Materials and Methods), larger than 0.05 ppm at a molar ratio of 1:3 can only be explained by dissociation constants exceeding high millimolar values, which are not easy to reconcile with a specific interaction.

Possibly the most important result of this study is that hL-FABP was not able to transfer its lipid cargo to *Pf*-UIS3(130-229). It can also be concluded

that direct docking to UIS3 is not modulated by the presence or absence of lipid molecules bound to hL-FABP. Clearly, these observations may not be used to draw definitive conclusions about *in-vivo* lipid transfer possibilities as additional cellular factors may intervene to activate such mechanisms. For example, the PVM may offer a suitable environment for protein-protein interaction or ligand release. Indeed, it has been shown that in close-to-physiological ionic strength conditions hL-FABP releases lipid molecules to a target membrane by a diffusion-based mechanism without direct collision.¹¹ Thus, a lipid-loaded hL-FABP may simply deliver its cargo to the PVM without the requirement to interact with membrane lipids or membrane-bound proteins such as UIS3. In support of this alternative hypothesis is the fact that the putative cytosol-exposed soluble domain of *Pf*-UIS3 did not show lipid binding capability in solution. Interaction of *Pf*-UIS3 with phospholipids, as shown in a crystal structure,¹⁷ appears to be a rather fortuitous event, made possible by crystal packing forces. Indeed, while one acyl chain of phosphatidylethanolamine adapts nicely to a shallow pocket on the *Pf*-UIS3 surface, the second chain departs from the protein molecule and interacts with a second protein molecule in its proximity in the crystal lattice.

The present results further suggest that a deeper investigation of the mouse system could be of

Figure 3. Interaction between *Pf*-UIS3(130-229) and lipid molecules. A: The ^1H , ^{15}N -HSQC spectrum of $[^{15}\text{N}]$ -*Pf*-UIS3(130-229) in the absence (black) and presence (orange) of a three-fold molar excess of oleate is shown. B: The ^1H , ^{15}N -HSQC spectrum of $[^{15}\text{N}]$ -*Pf*-UIS3(130-229) in the absence (black) and presence (orange) of a 10-fold molar excess of 1-palmitoyl-2-oleoyl-phosphatidylcholine is shown. The spectra in black have been offset low-field by 0.05 ppm (^1H) and upfield by 0.3 ppm (^{15}N). A schematic drawing of the molecules present in solution is displayed on the right of each panel.

value. hL-FABP and mL-FABP display strong sequence identity (84%) and it could be expected that they behave similarly. On the other hand the moderate sequence identity (34%) of *Pf*-UIS3 and *Py*-UIS3 could reveal system-dependent behaviors or substantiate the validity of the mouse model for the investigation of *P. falciparum* liver stage development.

Materials and Methods

Protein production

The cDNA encoding for the UIS soluble domain (residues 130-229) was PCR amplified using as template the *Pf*-UIS(83-229)-pGEX4T1 vector (gift from Prof. Sharma, International Centre for Genetic Engineering and Biotechnology, New Delhi, India). The result-

ing cDNA fragment was subcloned into BamHI and HindIII restriction sites of pQE50 vector carrying a C-terminal His₆-tag sequence. The sequence of the inserted cDNA was verified by plasmid sequencing.

The protein expression was performed using *E. coli* host strain SG (Qiagen). The starter cultures prepared in LB were inoculated into 1 l of Luria-Bertani broth or M9 minimal medium provided with $^{15}\text{NH}_4\text{Cl}$ as a sole nitrogen source for the expression of unlabeled or ^{15}N -labeled *Pf*-UIS(130-229) protein, respectively. Cells were grown at 37°C under continuous shaking till OD₆₀₀ was 0.6. Heterologous protein expression was induced by adding isopropyl- β -D-1-thiogalactopyranoside (IPTG) to a 1 mM final concentration. The cells were harvested 5 h after the induction. Cell pellets were resuspended in buffer containing 20 mM Tris, 500 mM NaCl, 0.02% NaN₃, pH 7.5, added with phenylmethanesulfonylfluoride (PMSF) as protease inhibitor and lysozyme. The pellets were incubated for 30 min. After sonication and centrifugation the supernatant containing the soluble *Pf*-UIS(130-229) was loaded onto a Ni-NTA column. Purification and elution were performed with buffer containing increasing amounts of imidazole. Tag cleavage was achieved using thrombin. A further purification step was carried out using size exclusion chromatography in 10 mM phosphate buffer, pH = 6.5, 0.02% NaN₃. Where indicated, a final delipidation step was performed as previously described.²⁴

Since the expressed protein was mostly present as insoluble inclusion bodies, a refolding protocol was optimized. The pellets were solubilized in a buffer containing 8M urea, 50 mM Tris, 500 mM NaCl, 0.02% NaN₃, pH = 8.0, and incubated at 10°C overnight under stirring to achieve complete resuspension. The following day, insoluble material was removed by centrifugation. The unfolded *Pf*-UIS(130-229) was refolded by 100-fold dilution in buffer containing 20 mM Tris, 500 mM NaCl, 0.02% NaN₃, pH = 7.5. Two dialysis steps were performed to complete the refolding of the protein. Purification steps were performed as described above for the soluble protein.

hL-FABP was expressed and purified as elsewhere described.^{16,25}

Unlabeled lysozyme from chicken egg-white was purchased as lyophilized powder from Sigma Aldrich.

The purity of all protein samples was verified by SDS-PAGE and NMR.

NMR experiments

All NMR experiments were recorded at 25°C on a Bruker Avance III spectrometer operating at 600.13 MHz proton Larmor frequency, equipped with a triple-resonance TCI cryoprobe incorporating a z-axis gradient. Typical 2D ^1H , ^{15}N -HSQC spectra were recorded using a standard pulse sequence, with a data matrix consisting of 2048 (F2, ^1H) $\times$ 256 (F1, ^{15}N) complex points. The number of scans

was 16 and the relaxation delay 1.5 s. Spectral windows of 9615 (F2) and 2189 (F1) Hz were used.

Processing of all the spectra was performed using Topspin 2.1 (Bruker).

The protein samples were typically 0.5 mM in protein, 10 mM phosphate buffer pH = 6.5, 0.02% NaN₃, added with 7% D₂O for spectrometer frequency lock.

Titration experiments were performed acquiring a series of ¹H, ¹⁵N-HSQC experiments at different protein–protein and protein–ligand molar ratios. The chemical shift changes of cross-peaks were determined as weighted averages according to the following equation:

$$\Delta\delta_{\text{HN}} = \{[(\Delta\delta_{\text{H}})^2 + (\Delta\delta_{\text{N}}/5)^2]/2\}^{0.5}$$

where $\Delta\delta_{\text{H}}$ is the chemical shift difference in the proton dimension and $\Delta\delta_{\text{N}}$ is the chemical shift difference in the nitrogen dimension. A value of $\Delta\delta_{\text{HN}} < 0.05$ ppm was considered non-significant.

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