

# Binding Analysis of a Novel Peptide to *Plasmodium falciparum* Knob-Associated Histidine Rich Protein (KAHRP)

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Knob-associated histidine rich protein (KAHRP) is secreted by *Plasmodium falciparum* in infected red blood cells. This protein is required for the production of surface protrusions called knobs, which have been shown to be crucial for the adherence of *P. falciparum*-infected erythrocytes (*Pf*-IRBC) to the endothelia of small blood vessels. KP-AP, a 10-amino acid (AA) peptide (FITRANDTSK), binds specifically with KAHRP in preliminary studies. KP-AP is expected to disrupt knob formation and prohibit adherence of *Pf*-IRBC to blood vessels and greatly reduce the pathogenicity of the parasite. This paper describes an investigation into the binding interaction between biotinylated KP-AP (Biotin-AP) and a segment of KAHRP. ELISA and the real-time bio-interaction optical sensor, BIAcore are the methods of detection. The specific binding was confirmed with ELISA and the KD value was estimated to be 1.2  $\mu$ M. Binding was not detected with BIAcore, most likely due to the reduced flexibility of Biotin-AP while immobilized on the sensor chip.

## Introduction

The high mortality of *Plasmodium falciparum* malaria is due to the presence of knobs at the surface of erythrocytes infected with *Plasmodium falciparum*. The formation of surface protrusions (knobs) in *P. falciparum*-infected erythrocytes is an important factor in the development of cerebral malaria.<sup>1,2</sup> Knob formation is associated with the synthesis of knob-associated histidine-rich protein (KAHRP) in ring and early trophozoite stages of the parasite. An important virulence factor, antigenic variant encoded *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is used by a parasite to interact with the human host and appears to be concentrated on the exterior surface of knobs with its c-terminal domain exposed to the erythrocyte cytoplasm, where parasite proteins are present, including KAHRP. The knobs facilitate adhesion of the *Pf*-IRBC to the endothelia of small blood vessels in vital organs by providing an elevated structure for the concentration of the adhesive ligand *Pf*-EMP1.<sup>3</sup> This cytoadhesion allows the parasite to escape destruction by the spleen.<sup>1</sup> It also plays a major role in the pathogenicity of the disease by causing the occlusion of small blood vessels and contributing to organ failure.<sup>4</sup>

Knob-associated histidine rich protein (KAHRP; Figure 1) is the main structural component of malaria knobs.<sup>2</sup> KAHRP assembles on the cytoplasmic face of the erythrocyte plasma membrane.<sup>2</sup> Knob formation and cy-

toadherence have been studied in vivo by the generation of cell lines with mutated versions of KAHRP. It has been shown that mutant malaria parasites lacking functional KAHRP are unable to bind to the inner membrane of blood vessels, regardless of whether the adhesive ligand *Pf*-EMP1 is being produced and trafficked to the cell surface.<sup>2,5</sup>

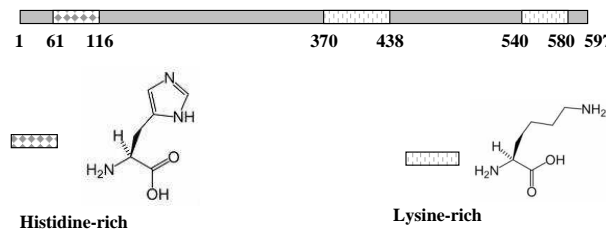


FIG. 1: Schematic representation of the full-length KAHRP protein. Amino acid residue numbers are shown adjacent to the KAHRP fragments in the protein schematic diagram.

KAHRP is a 654 AA g protein with a histidine-rich region towards the N-terminus and a long region of highly charged repeats towards the C-terminus which are referred to as the 3' and 5' repeats. KAHRP also contains signal sequences for its transport through the host cell.<sup>1</sup> The histidine-rich region interacts with the parasitic cell sorting machinery.<sup>2</sup> The 5' repeater region may be involved in cross-linking to the host cell cytoskeleton.<sup>2</sup>

KAHRP is believed to self-assemble due to the attraction of the positively charged histidine-rich region near the C-terminus and negatively charged lysine residues near the N-terminus.<sup>2</sup>

In this study, a peptide named KP-AP (FI-TRANDTSK) was investigated to determine whether it can disrupt the formation of knobs in *Pf*-IRBC by binding to a segment of KAHRP. KP-AP was discovered by the random generation of a 10 AA phage display library which was allowed to interact with *Pf*-IRBC in vitro. The specific binding between KP-AP and KAHRP was confirmed in preliminary studies using fluorescent microscopy.

## Methods

### ELISA

ELISA (The Enzyme-Linked ImmunoSorbent Assay) is a biochemical technique often used to detect the presence of an antigen or antibody in a sample.<sup>6</sup> In this experiment ELISA was used to detect binding between KAHRP and Biotin-AP. ELISA begins with the immobilization of an antigen on the polystyrene wells of an ELISA plate via adsorption. All non-specific binding sites are then blocked with a blocking reagent. After washing, various concentration of an antibody are added and allowed to form a complex with the antigen. The antigen-antibody complex can be detected only if the antibody is covalently linked to a reporter enzyme or if a secondary antibody is then added which is linked to a reporter enzyme through bioconjugation. After the antigen-antibody complex is linked to a reporter enzyme, a substrate is added to each well to develop a quantitative colorimetric signal, which is linearly proportional to the amount of bound antigen and antibody remaining after washing. The colorimetric signal is detected by an ELISA plate reader at a specific wavelength. The intensity of the signal correlates to the concentration of the bound sample-antibody complex. The results are graphed as optical density (OD) vs. concentration of the antibody. Optical density is a measure of the absorbance of the sample divided by the distance of the light path.

The dissociation constant ( $K_D$ ) of KAHRP and Biotin-AP can be roughly estimated using ELISA.  $K_D$  is an equilibrium constant that measures the tendency of a complex to fall apart into its component molecules.  $K_D$  has the dimension of concentration and is described by the following equation:

$$K_D = \frac{[A][B]}{[AB]} \quad (1)$$

Where  $[AB]$  is the concentration of the complex and  $[A]$  and  $[B]$  are the concentrations of the individual antigen and antibody, respectively.  $K_D$  can be roughly estimated from ELISA data by considering that there is some con-

centration of the molecules  $A$  and  $B$  that when at equilibrium, half of antigen  $A$  will be complexed with antibody  $B$  and therefore  $[A] = [AB]$ .<sup>7</sup> Substituting this into the Equation 1 gives:

$$K_D = \frac{[AB][B]}{[AB]} \quad (2)$$

$$K_D = [B] \quad (3)$$

Therefore, when half of antigen  $A$  is complexed with antibody  $B$ , the concentration of free antibody  $B$  is equal to the dissociation constant.<sup>7</sup> An estimate of  $K_D$  can be made by locating the point on the ELISA graph where half of the antigen is complexed with antibody, located in the center of the sigmoid-shaped curve. The concentration of antibody at that point is a rough estimate of the  $K_D$  value.

### BIAcore

The BIAcore instrument is used for the analysis of bio-specific interactions. It can provide real-time measurements of binding affinity.<sup>8</sup> Molecular interactions are indirectly detected by a diode which senses light reflected off of a gold-plated sensor chip. The sensor chip contains four separate flow cells (FC) with buffer solution flowing through them. One component of the interaction under study is immobilized on the sensor chip surface; this is referred to as the ligand. The other component, the analyte, flows through a single flow cell. The machine detects molecular interactions using the optical phenomenon of surface plasmon resonance (SPR). SPR occurs when light under conditions of total reflection interacts with a metal surface, causing an evanescent wave of oscillating electrons in the metal.<sup>8</sup> SPR is detected by a photodiode as a drop in light intensity. The intensity drop occurs at an angle which is affected by the refractive index very near the surface of the chip (less than one micrometer).<sup>8</sup> This angle is called the SPR angle. When an analyte solution flowing through a flow cell binds to a ligand immobilized on the gold-plated sensor chip, the change in refractive index near the chip surface causes a change in the SPR angle. This angle is recorded by the BIAcore computer and converted into resonance units (RU), which increase with increasing refractive index. 1000 RU is approximately equal to a change in the SPR angle of  $0.1^\circ$ , which correlates to roughly  $1 \frac{ng}{mm^2}$  change in surface protein concentration.<sup>8</sup> The binding data is displayed on a sensorgram with time on the x-axis and resonance units on the y-axis. Kinetic association and dissociation rate constants can in principle be derived from a plot of  $\frac{d(RU)}{dt}$  against  $RU$ :<sup>8</sup>

$$\text{slope} \left( \frac{d(RU)}{dt} \text{ vs. } (RU) \right) = k_{ass}C + k_{diss} \quad (4)$$

Where  $k_{ass}$  is the kinetic association constant,  $k_{diss}$  is the kinetic dissociation constant, and  $C$  is concentration.

The dissociation constant can be determined from the dissociation curve:<sup>8</sup>

$$\ln \left( \frac{RU_{t_1}}{RU_{t_n}} \right) = k_{diss} (t_n - t_1) \quad (5)$$

Where  $t_1$  is the initial time and  $t_n$  is any time during the dissociation phase.

A BIAcore assay involves three steps: immobilization, interaction, and regeneration. The immobilization of a ligand to the carboxymethylated dextran hydrogel layer of the sensor chip is accomplished by injecting the appropriate chemicals, depending on the type of biomolecule to be immobilized, through the flow cell. Often, activation and deactivation steps are necessary to facilitate ligand immobilization. After immobilization of the ligand, the analyte solution is injected into the flow cell and allowed to interact with the ligand for a specific amount of time. Finally, for the regeneration step, the sensor chip is washed by the injection of an appropriate solvent, leaving the immobilized ligand which can be reused.<sup>8</sup>

## Experimental

In this study, various regions of KAHRP are used. KP-N2 is a segment near the amino terminal; it is used in the BIAcore analysis. KP-C1 is a region within the KP-N2 segment; it is used in the ELISA.

The KAHRP fusion proteins were produced by ligation of the PCR-amplified KAHRP gene into a target vector (a plasmid), and inducing the vector into *E. coli* bacteria for genetic expression. The target vector also contained the genes for one of three tag proteins adjacent to the KAHRP gene: thioredoxin (TRX), glutathione-S-transferase (GST), or histidine (HIS), resulting in the production of the KAHRP fusion proteins: TRX-N2, GST-N2, and HIS-N2. The protein tags allowed for the purification of KAHRP from the bacterial proteome.

Biotin-AP was synthesized by machine. It consists of the KP-AP peptide with a 4 AA linker chain (SGSG) attached to increase flexibility. A biotin molecule was attached to the linker chain to facilitate immobilization on the sensor chip in the BIAcore analysis and conjugation with streptavidin in the ELISA.

## ELISA

For the ELISA, a polystyrene plate was used. Twenty-four wells were coated with 100  $\mu$ g glutathione-S-transferase (GST) as a negative control, and twenty-four wells were coated with 100  $\mu$ g GST conjugated KP-C1 (GST-C1). The plate was allowed to incubate for 3 hours at room temperature in a sealed container with a moist paper towel and then each well was washed three times with 1X Phosphate Buffered Saline (PBS) and two times with 0.1% PBS-Triton (PBS-T). 1X PBS was prepared by diluting 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub>,

and 0.24 g of KH<sub>2</sub>PO<sub>4</sub> in 1000 mL of deionized water and adjusting the pH to 7.4. PBS-T is composed of 0.1% Triton X-100 in 1X PBS. After washing, all nonspecific binding sites were blocked by adding 2% bovine serum albumin (BSA) in 0.1% PBS-T buffer and incubating at 4 °C overnight. The washing with PBS and PBS-T was repeated and then the wells were coated with various concentrations (0, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0  $\mu$ M) of Biotin-AP or the negative control Biotin-SP (scrambled peptide) (0, 0.5, 1.0, 2.0, and 4.0  $\mu$ M) and allowed to incubate for 3 hours at room temperature. After another washing with PBS and PBS-T the second antibody, 150  $\mu$ L streptavidin horseradish peroxidase (SA-HRP) 1:5000 in 2% BSA, was added and allowed to incubate for 2 hours at room temperature. The streptavidin bound to any biotin present in the ELISA plate. Finally the substrate, 100  $\mu$ L per well of prepared solution from the Pierce Peroxide Solution Kit, was added and allowed to incubate for 30 minutes at room temperature, producing a blue color in some of the wells. The reaction was stopped with 2.0 M sulfuric acid and the absorbance was read in an ELISA plate reader at 450 nm.

## BIAcore

The sensor chip used was an SA type which is coated with streptavidin which binds strongly to biotin, ensuring simple immobilization in the proper configuration. The running and sample buffer used was HEPES Buffered Saline pH 7.4 (HBS), which was prepared by diluting 2.38 g HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid), 8.77 g NaCl, 1.27 g EDTA, and 0.5 ml BIAcore P20 surfactant in 1000 mL deionized water and adjusting the pH to 7.4. The solution was filtered through a 0.22  $\mu$ m filter, degassed, and stored at room temperature.

The flow rate for the BIAcore immobilization and analysis was 5  $\frac{\mu\text{L}}{\text{min}}$ . For the immobilization phase, 35  $\mu$ L of 300 nM Biotin-AP in HBS was allowed to flow through FC 2 for 5 minutes. FC 1 was kept empty to be used as a negative control. For the analysis, 15  $\mu$ L of each of the three fusion proteins (2000 nM each in HBS) were injected into both FC 1 (the negative control) and FC 2 (the peptide-coated sensor chip) for 3 minutes at 25 °C.

## Results

### ELISA

The ELISA indicates a specific binding interaction between GST-C1 and Biotin-AP (Figure 2). The  $K_D$  value is roughly 1.2  $\mu$ M, indicating a moderately strong interaction. Ideal  $K_D$  values for drugs are in the nanomolar range.<sup>9</sup>

The negative controls used indicated that the binding between GST-C1 and KP-AP is specific. The low opti-

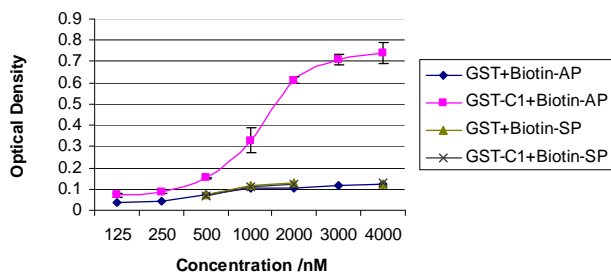


FIG. 2: Absorbance readings of various KP-AP concentrations interacting with GST-C1 coated wells. The optical density readings at 450 nm are plotted against various concentrations of Biotin-AP or Biotin-SP. Twenty-four wells were coated with 100  $\mu\text{g}$  glutathione-S-transferase (GST) as a negative control and twenty-four wells were coated with 100  $\mu\text{g}$  GST-C1. Antibodies used were various concentrations (0, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0  $\mu\text{M}$ ) of Biotin-AP and the negative control Biotin-SP (scrambled peptide) (0, 0.5, 1.0, 2.0, and 4.0  $\mu\text{M}$ ). Streptavidin horseradish peroxidase (SA-HRP) was the second antibody (150 per well of a 1:5000 dilution in 2% BSA), and peroxide was the substrate (100  $\mu\text{L}$  per well of Pierce peroxide substrate).

cal density readings from wells with GST and Biotin-AP indicate that GST does not bind strongly to Biotin-AP; the low optical density readings from wells with GST and Biotin-SP (scrambled peptide) indicate that GST does not bind strongly to biotin; the low optical density readings from the wells with GST-C1 and Biotin-SP indicate that KP-C1 does not bind strongly to biotin.

The sigmoid shape of optical density readings from the wells containing GST-C1 and Biotin-AP is typical of ELISA graphs. The upper plateau indicates that GST-C1 became saturated with Biotin-AP. The optical density did not begin to increase dramatically until the concentration of GST-C1 was around 500 nM, implying a somewhat weak interaction. The low OD values from the three negative controls indicate that the binding between KP-C1 and KP-AP peptide is specific.

### BIAcore

The peptide was successfully immobilized on the ligand, as indicated by Figure 3. The binding analysis in FC 2 showed data similar to the negative control in FC 1 (Figures 4 and 5); there was no detectable binding between Biotin-AP and the KAHRP fusion proteins used.

The immobilization of Biotin-AP onto the sensor chip is observable in Figure 3. The immobilization phase occurs between 150 s and 600 s. The large difference between the baseline at 0 s and the flat portion after 600 s indicates that Biotin-AP remained on the chip after the 300 nM solution was washed off with buffer. The steep slope during immobilization indicates that there was a strong interaction between the biotin in the Bio-AP and

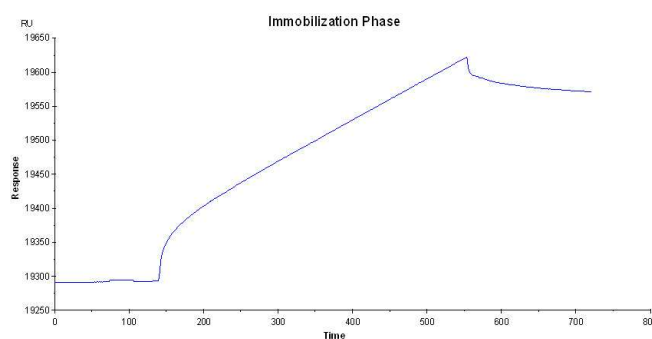


FIG. 3: BIAcore sensorgram for the immobilization of Biotin-AP. 35  $\mu\text{L}$  of 300 nM Biotin-AP in pH 7.4 HBS was injected into flow cell 2 (FC 2) of a streptavidin (SA) coated sensor chip at a rate of 5  $\frac{\mu\text{L}}{\text{min}}$  for 5 min at 25  $^{\circ}\text{C}$ . The immobilization process occurs between 140 s and 550 s.

the streptavidin-coated sensor chip. The fact that the slope does not reach a plateau before the bio-pep was washed off with buffer at 550 s indicated that there were still some open binding sites available on the SA chip. The overall change was 280 response units (RU), which correlates to 0.3  $\frac{\text{ng}}{\text{mm}^2}$  Biotin-AP.

Each injection of KAHRP fusion protein into the negative control flow cell (FC 1) is seen in Figure 4 as a steep rise which levels off and then drops down again. The injections begin at 180 s (TRX-N2), 560 s (GST-N2), and 910 s (HIS-N2). The steep rise at the beginning of each injection results from a change in ionic strength between the sample buffer and the HBS. The flatness of the curve during the injections indicates a weak interaction between the polymer surface of the sensor chip and the fusion proteins. If there was a strong binding interaction, the curves would have had a larger slope and appear as in Figure 3. For the binding analysis, seen in Figure 5, the

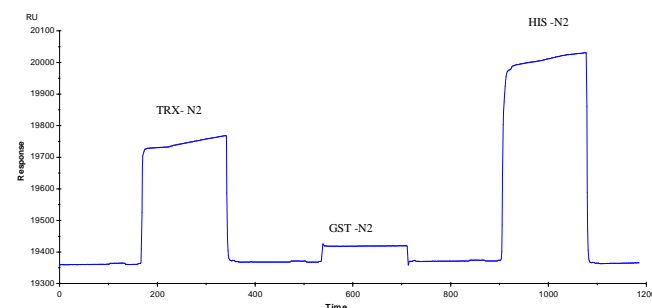


FIG. 4: BIAcore sensorgram for the negative control analysis of 15  $\mu\text{L}$  200 nM KAHRP fusion proteins. Each curve shows the injection of 15  $\mu\text{L}$  of a 200 nM KAHRP fusion protein (from left to right: TRX-N2, GST-N2, and HIS-N2) diluted in HBS into flow cell 1 (FC 1) at a flow rate of 5  $\frac{\mu\text{L}}{\text{min}}$  for 3 min at 25  $^{\circ}\text{C}$ . FC 1 is an empty cell used as a negative control.

injections begin at 115 s (TRX-N2), 600 s (GST-N2),

and 1110 s (HIS-N2). Again, the steep rise at the beginning of each injection resulted from a change in ionic strength between the sample buffer and the HBS. The flatness of the curve during the injections represents a weak interaction between the Biotin-AP immobilized on the sensor chip and the KAHRP fusion proteins. If there was a strong binding interaction, the curves would have had a larger slope and appear as in Figure 3.

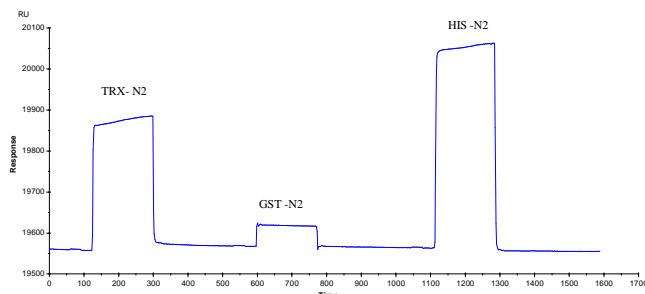


FIG. 5: BIAcore sensorgram for the analysis of the interaction between 15  $\mu\text{L}$  of the 200  $n\text{M}$  KAHRP fusion proteins and the biotin-peptide-coated sensor chip. Each curve shows the injection of 15  $\mu\text{L}$  of a 200  $n\text{M}$  KAHRP fusion protein (from left to right: TRX- N2, GST-N2, and HIS-N2) diluted in HBS into FC 2 at a flow rate of 5  $\frac{\mu\text{L}}{\text{min}}$  for 3 min at 25  $^{\circ}\text{C}$ . The sensor chip surface of FC 2 contained immobilized Biotin-AP.

## Discussion

The specific binding interaction between a KAHRP fusion protein and Biotin-AP was clearly observable with ELISA, but was unable to be detected with the BIAcore machine under the experimental conditions used. BIAcore is a very sensitive instrument and there are several important factors to consider when planning an experiment. Every experimental parameter must be chosen with great care, as small changes in size and concentration cause large differences in the BIAcore response. If a protein is of the wrong size or concentration, binding interactions are not always detectable. For a successful assay, trial and error is usually necessary.

KAHRP Fusion Protein	Flow Cell 1 /RU	Flow Cell 2 /RU
TRX-N2	407	352
GST- N2	58	56
HIS- N2	670	503

TABLE I: Comparison of Fusion Protein Binding Response in Flow Cells 1 and 2 The relative RU's from the baseline to the top of the curve for each fusion protein is shown. The smaller RU values from FC 2, versus the negative control FC 1, indicate that the fusion proteins did not have a strong interaction with the Biotin-AP.

Biotin-AP is a very small molecule. The delectability limits of the BIAcore machine required a high concentration of Biotin-AP on the BIAcore sensor chip. This was facilitated by the strong interaction between biotin and the streptavidin-coated sensor chip. The high concentration of Biotin-AP immobilized on the sensor chip ability may have been reduced due to steric hindrance and decreased diffusion of KAHRP fusion proteins into the Biotin-peptide layer. Although a linker peptide (SGSG) was added to increase flexibility, this may not have been sufficient to provide access of KAHRP to the immobilized peptide. The ELISA results indicate that the binding interaction is moderately weak, which further decreased the possibility of detecting binding with BIAcore under the conditions used.

KAHRP and KP-AP have a moderately weak attraction to each other, as shown by the ELISA data. The binding of KAHRP and a peptide similar to KP-AP may disrupt the formation of knobs in *P. falciparum*-infected parasites, but a better understanding of the binding mechanism is needed. An alanine knockout experiment will need to be performed to probe the binding mechanism. In this experiment, each amino acid of KP-AP will be replaced with alanine, one at a time, and the binding interaction will be studied with ELISA. The relative binding ability of the various alanine knockout peptides will determine the important residues involved in binding and may lead to the discovery of a new malaria drug.

## Conclusion

Because KP-AP is a very small peptide, its binding ability was greatly reduced when attached to the BIAcore sensor chip. The high concentration of Biotin-AP on the sensor chip surface caused steric hindrance and restricted the access of KAHRP into the Biotin-AP layer. The short linker region of the peptide may have been insufficient at providing flexibility to such a small molecule.

Binding between KP-AP and the C1 segment of KAHRP is specific, as observed in the ELISA results. The strength of the interaction is moderately, strong, with an estimated  $K_D$  value of 1.2  $\mu\text{M}$ .

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