Dendrimeric Template of *Plasmodium falciparum* Histidine Rich Protein II Repeat Motifs Bearing Asp→Asn Mutation Exhibits Heme Binding and β-Hematin Formation

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Rationale

Understanding heme polymerization mechanism is the first step for rational design of new drugs, targeting this pathway. Heme binding and hemozoin formation have been ascribed to PfHRPII aspartate carboxylate-heme metal ionic interactions. To investigate, if this ionic interaction is indeed pivotal, we examined the comparative heme binding and β-hematin forming abilities of a wild type dendrimeric peptide BNT1 (harboring the native sequence motif of PfHRPII (AHHAHHAADA)) versus a mutant dendrimeric peptide BNTM (in which Aspartate residues have been replaced by the neutral Asparaginyl residues (AHHAHHAANA)). UV and IR data reported here reveal that at pH 5, both BNT1 and BNTM exhibit comparable heme binding as well as β-hematin forming abilities, thus questioning the role of PfHRPII aspartate carboxylate-heme metal ionic interactions in heme binding and β-hematin formation. Based on our data and information in the literature we suggest the possible role of weak dispersive interactions like N-H⋯π and lone-pair-⋯π in heme binding and hemozoin formation.

Abstract

*Plasmodium falciparum* (Pf) employs a crucial PfHRPII catalyzed reaction that converts toxic heme into hemozoin. Understanding heme polymerization mechanism is the first step for rational design of new drugs, targeting this pathway. Heme binding and hemozoin formation have been ascribed to PfHRPII aspartate carboxylate-heme metal ionic interactions. To investigate, if this ionic interaction is indeed pivotal, we examined the comparative heme binding and β-hematin forming abilities of a wild type dendrimeric peptide BNT1 (harboring the native sequence motif of PfHRPII (AHHAHHAADA)) versus a mutant dendrimeric peptide BNTM (in which Aspartate residues have been replaced by the neutral Asparaginyl residues (AHHAHHAANA)). UV and IR data reported here reveal that at pH 5, both BNT1 and BNTM exhibit comparable heme binding as well as β-hematin forming abilities, thus questioning the role of PfHRPII aspartate carboxylate-heme metal ionic interactions in heme binding and β-hematin formation. Based on our data and information in the literature we suggest the possible role of weak dispersive interactions like N-H⋯π and lone-pair-⋯π in heme binding and hemozoin formation.

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Introduction

Malaria is one of the most severe public health problems worldwide. In 2010, malaria caused an estimated 216 million clinical episodes, and 655,000 deaths [1]. Children under five and pregnant women are the most affected groups. Five species of *Plasmodium* have been recognized to infect humans; of whom *Plasmodium falciparum* (Pf) is the most virulent and most recently evolved [2]. Antimalarial drug resistance and lack of new antimalarials are major challenges to malaria control.

Toxic heme constitutes the inevitable outcome of the penchant of the malaria parasite to digest hemoglobin for its nutritional requirements in the blood stage of its life cycle. This process which occurs in the miniscule volume of a few femtoliters in the food vacuole of the parasite leads to a massive accumulation of heme leading to concentration as high as 300–500 mM [3]. The highly successful proliferative life cycle of the malaria parasite notwithstanding such a toxic milieu, owes a lot to the efficient heme detoxification strategies evolved by the parasite. One such strategy involves the deployment of “heme mopper proteins” such as the histidine rich proteins (HRP) II and III [4], which are characterized by the occurrence of repetitive histidine rich heme binding sequence motifs; that occur throughout the sequence of these natively unfolded proteins. Hemozoin is apparently identical to β-hematin (an *in vitro* synthesized complex of heme), which is a reciprocal dimer of Fe(III)PPIX in which propionate groups of each heme coordinates with the Fe(III) centre of the neighboring heme and the resulting dimers are linked through hydrogen bonds across the second propionic acid groups [5]. Malarialogists and drug developers have been fascinated by this pathway of heme detoxification since it is absent in humans. Currently two hypotheses concerning hemozoin formation viz via proteins like the Histidine rich protein (HRP) and the heme detoxification protein (HDP) [6] or via the hydrophobic milieu provided by lipids are in vogue [7]. However the exact mechanism of hemozoin formation in *P. falciparum* still remains unclear [8] and in particular the role of HRPs in hemozoin formation is still unknown and open to investigation.

PfHRPII is a 30 kDa protein, 85% of which consists of repeat sequences like Ala-His-His-Ala-His-Ala-Ala-Ala-Asp-Ala [9,10]. PfHRPII has been shown to bind Fe(III)PPIX with significant changes in its conformation [11,12]. While synthetic linear peptides corresponding to these sequence motifs show heme binding but no hemozoin formation, K-K2 dendrimers called bionucleating templates (BNTs) exhibiting four branches of this linear motif have been shown to not only bind heme but also catalyze β-hematin formation [13]. At food vacuole pH (4.5–5.5) PfHRPII-heme binding has been assumed to be via aspartate carboxylate-metal ionic/coordinate interactions and hemozoin...
formation has been correlated with aspartate binding [12]. In the present work, we wanted to study if aspartate carboxylate-heme metal ion interactions are indeed crucial to heme binding and \( \beta \)-hematin formation. Towards this, we have replaced the four aspartyl residues of BNT1 by asparagine residues to obtain BNTM (Figure 1). Here we report that metal ion interactions are not essential since both BNT1 and BNTM exhibit comparable heme binding as well as \( \beta \)-hematin forming abilities.

### Materials and Methods

#### Materials and Sample Preparation

All solvents and reagents were used as received unless specified. Fmoc (Fluorenlymethylcarbonyl) amino acid derivatives {Fmoc-Ala-OH, Fmoc-Asn (otBu), Fmoc-His (trt), Rink Amide methylbenzhydrylamine (\{100–200 mesh\}, MBHA)} resin and O-Benzotriazole-N, N', N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) for peptide synthesis were from Nova Biochem. Fmoc-Glycine and Fmoc-L-Lys-(Fmoc)-OH were from Chem-Impex International and Orpegen Pharma respectively. Dimethylformamide (DMF), Piperidine, N,N'-Disopropylcarbodiimide (DIPCDI), 1-Hydroxy Benzotriazole Monohydrate (HOBT), N,N'-Disopropylcarbodiimide (DIEA), Trifluoroacetic acid (TFA), Trisopropylsilane (TIS), Dimethyl sulfoxide (DMSO), Heme, HEPES and Polyhistidine were from Sigma. Acetonitrile (ACN) was from J. T. Baker. General reagents used were of analytical grade.

Fresh stock solution of heme was made by dissolving recrystallized heme in DMSO and the solution was filtered through a 0.45 \( \mu \)m syringe filter. Concentration of the resulting solution was determined spectrophotometrically in DMSO using an extinction coefficient of 170 m\(^{-1}\) cm\(^{-1}\) at 404 nm [14]. BNT1 and BNTM stock solutions were made in 20 mM sodium acetate (pH 5), and stored at –20°C.

#### Synthesis of Bionucleating Peptide Dendrimer Templates

K-K2 peptide dendrimer templates (BNT1 and BNTM) were synthesized as C-terminal amides using standard Fmoc chemistry on Rink amide MBHA resin in manual mode using Fmoc amino acid/HBTU/HOBT/DIEA in the molar ratio of 1:1:1:2 in DMF. Fmoc-Lys (Fmoc)-OH was used to create a branch core for the synthesis of the K-K2 peptide dendrimers. Both Fmoc groups of Fmoc-Lys (Fmoc)-OH-resin complex were removed by treatment with 20% piperidine in DMF giving rise to two (\( \alpha \) and \( \varepsilon \)) amino group. The synthesis of peptide dendrimers was accomplished on a K-K2 core generated by coupling of Fmoc-Lys (Fmoc)-OH to the two free amino groups on the lysine coupled resin synthesized as described above. Four free amino groups (\( \alpha \) and \( \varepsilon \), for each lysine derivative) so obtained, were used for simultaneous synthesis of four identical chains of the same peptide as shown in Figure 1.

### Stability of BNTM

BNTM (100 \( \mu \)M) in 100 \( \mu \)L of buffer (500 mM sodium acetate, pH 5) was incubated under \( \beta \)-hematin forming conditions (260 rpm, 24 hrs, and 37°C). The treated sample of BNTM (BNTM (\( t \))) was diluted to 1 mL with 0.5% acetic acid and passed (2 times) through C\(_{18}\) mini spin column (\{Pierce\}, equilibrated with 0.5% acetic acid). Column was washed with 0.5% acetic acid and elution of BNTM (\( t \)) was done with 80% acetonitrile solution. After evaporation and lyophilization, solid BNTM (\( t \)) was analyzed by ESI-MS.

### Titrimetric determination of heme binding sites

Difference spectroscopy (heme titrations) studies were done as described earlier [16]. Spectra were recorded on a Perkin-Elmer Orbitrap velos pro (Thermo Fisher Scientific).

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Figure 1. Sequence of dendrimeric peptides used in this study. All aspartic acid (D) residues of BNT1 have been substituted by asparagines (N) in BNTM. \( \alpha \) and \( \varepsilon \) denote amino groups of lysine.

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Lambda Bio20 spectrophotometer between 250–700 nm at a speed of 120 nm/min and a slit width of 1 nm. Heme was titrated in two separate experiments, for binding to BNT1 and BNTM. A hemin solution (2 mM in DMSO) was simultaneously titrated into (i) a solution of 5 μM of BNT1/BNTM in 3 mL of 200 mM HEPES, pH 7 and (ii) a reference cuvette containing HEPES buffer only. Heme was titrated in 2 μM increments. After each addition of heme, the samples in the two cuvettes were mixed and allowed to stand for 5 minutes to allow complete binding before recording of difference spectra. Difference absorption spectra were recorded after each incremental addition of heme. Concentration of DMSO was kept below 2% in all reaction mixtures. The resulting mixture was centrifuged as above and supernatant was vortexed for 5 minutes and centrifuged (13000 rpm, 60 min, 25°C). Pellet so obtained was suspended in 1.5 mL of 100 mM sodium bicarbonate (pH 9.2) by vortexing, sonication, and centrifugation (13000, 15.7 rcf, rpm, 60 min, 25°C). Two additional bicarbonate washes were given as above. Pellets were washed twice with 2.5% SDS by resuspending, vortexing, sonication, and centrifugation (13000, 15.7 rcf, rpm, 60 min, 25°C). In order to characterize hemozoin by spectroscopy, pellets were resuspended in 500 μL of 2.5% SDS, vortexed for 5 min and incubated overnight. Next day spectra were recorded between 300 nm–700 nm.

Spectroscopic Characterization of Hemozoin

Fresh stock solution of heme was made by wetting recrystallized heme (Sigma) in 1N NaOH, diluting with 100 mM HEPES (pH 7). Resulting solution was vortexed, centrifuged (13000 rpm, 15 min, 25°C), supernatant filtered through 0.22 μm syringe filter and concentration of heme was determined spectroscopically in 100 mM HEPES (pH 7) using molar extinction coefficient at 400 nm of 62 M⁻¹ cm⁻¹. Heme (final concentration 50 μM) was aliquoted into eppendorf tubes. BNT1 and BNTM (peptide dendrimers, 20 nmoles) were added and finally the volume was brought to 1.5 mL with 500 mM sodium acetate buffer (pH 5). Heme alone (negative control) was also run simultaneously. Each assay was set up in quadruplicate and incubated at 37°C for 36 hours on a rotating shaker (260 rpm). The reaction was stopped by adding 10 μL of 10% SDS in PBS. Reaction mixture was vortexed for 5 minutes and centrifuged (13000 rpm, 60 min, 25°C). Pellet was resuspended in 1.5 mL of 100 mM sodium bicarbonate (pH 9.2) in 2.5% SDS by vortexing and sonication. The resulting solution was centrifuged (13000 rpm, 15.7 rcf, rpm, 60 min, 25°C). Two additional bicarbonate washes were given as above. Pellets were washed twice with 2.5% SDS by resuspending, vortexing, sonication, and centrifugation (13000, 15.7 rcf, rpm, 60 min, 25°C). In order to characterize hemozoin by spectroscopy, pellets were resuspended in 500 μL of 2.5% SDS, vortexed for 5 min and incubated overnight. Next day spectra were recorded between 300 nm–700 nm. Hemozoin suspensions were then made 0.02 N with NaOH to obtain typical hematin UV-visible spectrum in each case [18]. β-hematin (an in vitro synthesized complex of heme and apparently identical to hemozoin [19]) spectrum was also recorded under similar condition and used as reference.

FT-IR Spectroscopy

To obtain IR spectra, KBr pellets were prepared from dried samples of BNT1,BNTM, polyhistidine mediated heme aggregates and β-hematin spectra were acquired for 100 cycles with a Fourier-transform IR spectrometer (Perkin-Elmer).

Results and Discussion

Characterization of K-K2 Peptide Dendrimers (BNT1, BNTM) by RPHPLC and Mass Spectroscopy

BNT1 and BNTM were purified by RPHPLC using acetonitrile-water gradient. The observed retention times (min) was 18 and 17 for BNT1 and BNTM respectively (Figure S1). The identities of these two BNTs were confirmed by mass spectrometry. Observed mass values were 4703 Da for BNT1 (Expected mass- 4704 Da) and 4698 Da for BNTM (Expected mass-4700 Da) {Figure S2, panels A and B}.

Stability of BNTM

There is possibility of deamidation of asparagine moieties of BNTM to aspartate under conditions used to promote hemozoin formation. This could lead to in situ generation of BNT1 from BNTM. So stability of BNTM treated {BNTM (t)} under high ionic strength and acidic condition (500 mM sodium acetate, pH 5) was analyzed by mass spectrometry. The observed mass values for BNTM and BNTM (t) were 4698 Da and 4699 Da respectively {Figure S2, panels B and C}. This observed difference in mass of 1 Dalton between BNTM and BNTM (t) is most likely to be due to low MS resolution and not due to deamidation since 401 nm versus time were plotted and data were evaluated by standard deviation and t test.
selective hydrolysis of one out of four asparaginyl residue of BNTM is quite unlikely. The nearly identical masses of BNTM and BNTM (t) confirm the stability of BNTM under acidic and high ionic strength conditions used to promote hemozoin formation.

**BNT1 and BNTM: Spectroscopic titrimetric determination of heme binding sites and comparative kinetics of heme binding**

To examine if the synthetic peptide dendrimers bind with heme, we analyzed changes in the absorption spectrum of heme ($\lambda_{\text{max}}$ 384 nm) after mixing it with BNT1 or BNTM. The difference spectra (Figure 2, Panels A and B) showed the principal Soret peak at 415 nm for BNT1-heme and BNTM-heme complexes. The heme-binding sites on BNT1 and BNTM were titrated with increasing amounts of heme and difference absorption spectra were recorded at varying heme concentrations. Red shift ($\lambda_{\text{max}}$ 384 to 415 nm) was observed upon heme binding to BNT1/BNTM. The absorbance at 415 nm increased until it reached a plateau. Analysis of heme binding curves indicated binding stoichiometries of $5.80.6$ and $6.750.4$ for BNT1/BNTM: heme respectively (Figure 2, panel C). The BNT1-heme stoichiometry observed...
here is consistent with a previous study of BNT1-heme binding [13].

No significant difference was found in heme binding stoichiometries of BNT1/BNTM binding at 415 nm. Absorbance at 415 nm corresponds to the Soret peak of the stable bis-histidyl heme complex and does not provide direct information on Asp/Asn contributions to heme binding. To study the contribution of Asp/Asn in kinetics of bis-histidyl complex formation, comparative kinetics of heme binding potential of BNT1 vs BNTM was studied. As shown in (Figure 2, panel D) there is no significant difference in the kinetics of heme binding by these two peptides.

BNT1 vs BNTM: Comparative kinetics of hemozoin formation

Heme binding to a template protein is essential for formation of a supramolecular structure like hemozoin. However heme binding alone is not sufficient for β-hematin/hemozoin formation. For instance, polyhistidine binds multiple monomers of heme but fails to form β-hematin [4]. Changes in secondary structure of template protein like PfHRPII have been observed upon PfHRPII-heme binding [12,20], suggesting that favorable secondary structures on the protein template may be essential to promote hemozoin formation. Acidic pH (4.5–5.5) also seems to play a crucial role in

Figure 3. Spectroscopic characterization of bicarbonate stable heme aggregates formed in presence of BNT1/BNTM. Panels A, C and E show UV-visible spectra of β-hematin and BNT1/BNTM mediated bicarbonate stable heme aggregates respectively. These aggregates were incubated overnight in 2.5% aqueous SDS prior to recording spectra (blue line). Note the broad Soret (400 nm) and the weak but characteristic peak at 655 nm. Typical hematin spectra (sharp Soret at 400 nm - red line) were obtained when each suspension was made 0.02 N with respect to NaOH. Panels B, D and F show the FTIR spectra of β-hematin and BNT1/BNTM mediated bicarbonate stable heme aggregates respectively. Peaks at 1664 cm\(^{-1}\) and 1210 cm\(^{-1}\) in FTIR spectrum of heme aggregates mediated by BNT1 and BNTM confirm their identity as β-hematin.
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hemozoin formation as half of the propionate side chains of heme are deprotonated at this pH, thus allowing the formation of iron carboxylate metalloester bonds between propionate side chains of one heme with the central Fe\textsuperscript{3+} of another heme [19,5].

Difference spectroscopy of heme binding to BNT1 and BNTM motivated us to investigate their \( \beta \)-hematin forming abilities. Both BNT1 and BNTM led to \textit{in vitro} \( \beta \)-hematin formation when incubated with heme at pH 5 (Figures 2 and 3). Yield of \( \beta \)-hematin increased with increasing incubation time in case of both BNT1 and BNTM. Statistical analysis (p value) at each time point confirmed that there is no significant difference in the level of \( \beta \)-hematin formed by BNT1 vs BNTM (Figure 2, panel E) suggesting that aspartate (BNT1) and asparagine residues (BNTM) do not differentially impact hemozoin formation.

UV-Visible spectroscopy was done to examine if these bicarbonate stable BNT1/BNTM-heme aggregates have features of \( \beta \)-hematin [18]. Heme aggregates formed by BNT1/BNTM (Figure 3, Panels C and E) upon overnight incubation in 2.5% aqueous SDS, exhibited an absorption maximum at 655 nm and a broad Soret band centered at \( \sim 400 \) nm. When NaOH was added to these suspensions, the resulting solutions exhibited sharp Soret band centered at 400 nm typical of heme. The two absorbances (655 and 400 nm) have been considered as spectroscopic signatures associated with \( \beta \)-hematin [18] and their presence in panels C and E therefore suggests that the aggregates formed in presence of BNT1/BNTM are \( \beta \)-hematin. Peaks at 1664 cm\textsuperscript{-1} and 1210 cm\textsuperscript{-1} in FTIR spectrum [19] of heme aggregates mediated by BNT1 and BNTM further confirm their identity as \( \beta \)-hematin (Figure 3, Panels D and F).

The exact role of Aspartyl residues in \( P/H \text{HRPII} \) towards heme binding and hemozoin formation is unclear. Lynn et al [12] speculated that at pH \( \sim 5 \), aspartates of \( P/H \text{HRPII} \) may form ionic bonds with iron (Fe\textsuperscript{3+}) of heme. However, our observations reported in the present work show that BNTM in which Aspartyl residues have been replaced by Asparaginyl residues still binds heme and forms \( \beta \)-hematin (Figures 2 and 3). This suggests that iron-aspartate carboxylate ionic interactions may not be crucial for hemozoin/\( \beta \)-hematin formation. It is important to note that polyhistidine which binds multiple monomers of heme also gave rise to bicarbonate stable aggregates of heme. However these polyhistidine binding at pH 4.8 (500 mM acetate buffer) has no effect on either heme binding or \( \beta \)-hematin formation. Our findings suggest that weak dispersive intermolecular interactions may also play a role in stabilizing heme-template complex and inducing specific structural changes in the template required to initiate hemozoin formation.

Conclusions

Replacement of anionic Aspartyl residues in the template BNT1 (\textit{AHHAHAAAADA}) by the neutral Asparaginyl residues {BNTM, (\textit{AHHAHAAANA})} has no effect on either heme binding or \( \beta \)-hematin formation. Our findings suggest that weak dispersive forces like N-H\( \cdots \)p interactions may also play a role in stabilizing heme-template complex and inducing specific structural changes in the template required to initiate hemozoin formation.

Supporting Information

Figure S1 Characterization of BNT1 and BNTM by RPHPLC. Panels A and B show RPHPLC profiles of BNT1 and BNTM respectively. Analytical purity of Purified BNT1 and BNTM was assessed by chromatography on a reversed phase C\textsubscript{18} column (Microsorb, 15 x 4.6 mm, 15 \( \mu \) using 0.1% TFA/water (solvent A) and 0.1% TFA/acetonitrile (solvent B) and running a 5–45% linear gradient over 20 minutes at a flow rate of 1 mL/min. Wavelength detector was set at 214 nm. (TIF)

Figure S2 Mass Spectrum (ESI-MS) of peptide dendrimer BNT1 (panel A), BNTM (panel B) and BNTM (\( t \)) (panel C). (\( t \)) represents the sample of BNTM after it was treated under the acidic and high ionic strength conditions used to promote hemozoin formation (500 mM sodium acetate pH 5, 37\( ^\circ \)C, 24 hrs). Observed mass values for BNT1, BNTM and BNTM (\( t \)) were 4703 Da, 4696.43 Da and 4699.28 Da respectively. The difference of 4 Daltons between the observed masses of BNT1 and BNTM is as expected since replacement of four Aspartyl residues with four Asparaginyl residues is consistent with a loss of four Daltons. The nearly identical masses of BNTM and BNTM(\( t \)) suggests that 500 mM sodium acetate pH 5, 37\( ^\circ \)C, 24 hrs- the conditions used to promote hemozoin formation did not cause deamidation of Asparaginyl residues. (TIF)

Figure S3 UV-Visible spectra of heme and heme-polymhistidine complex (A) and FTIR spectra (B) of polyhistidine mediated bicarbonate stable heme aggregates. Panel A shows heme-polymhistidine binding at pH 4.8 (500 mM acetate buffer). Bathochromic shifts from \( \sim 384 \) to 415 nm indicate heme binding to
polyhistidine. Panel B shows comparison of FTIR spectra of β-hematin (black line), polyhistidine mediated heme aggregates (blue line) and heme (red line). Characteristic signatures peaks of β-hematin (1210 cm\(^{-1}\) and 1664 cm\(^{-1}\)) are absent in both polyhistidine mediated heme aggregates and heme.

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