Multiple-Antigenic Peptides of Histidine-Rich Protein II of *Plasmodium falciparum*: Dendrimeric Biomineralization Templates

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Abstract: A critical target for the development of new antimalarial treatments is the detoxification pathway of free heme released during the catabolism of host hemoglobin in the digestive vacuole of the malaria parasite *Plasmodium falciparum*. We have examined a family of peptide dendrimers (BNT I and II) based on the tandem repeat motif of HRP II from *P. falciparum* for their abilities both to bind heme substrates and to form the critical detoxification polymer hemozoin. Each template was capable of binding significant amounts of the natural substrate, Fe(III)PPIX. Binding of the metal-free base protoporphyrin IX to the templates suggests, however, that substrate recognition is based on the porphyrin moiety rather than specific metal recognition. Such a supposition is further supported by the fact that Zn(II)PPIX, as well as the structurally related metal-free and metallophthalocyanines, can bind to the templates. Further, it was shown that the dendrimeric BNT I and BNT II were capable of supporting the polymerization of hemozoin. In light of previously reported binding studies of linear sequences derived from HRP II and the polymer polyhistidine, the results suggest that the tandem organization of the tripeptide binding sites promotes the formation of hemozoin for these model templates.

As recently as forty years ago, only 10% of the world's population was at risk from malaria. Today, as a result of increased parasite resistance to traditional antimalarial drugs and vector resistance to insecticides, over 40% of the world's population is at risk. There is little doubt that malaria is once again resurgent. Current estimates place the number of malaria cases between 200 and 500 million worldwide, with over 2 million deaths annually. The economic strain placed on developing countries reached 2.0 billion dollars (US) annually in 1995 and is predicted to increase.[1] Consequently, the development of new treatments to combat drug-resistant strains of *Plasmodium* is critical.

Recent studies of the chemistry of the digestive vacuole (pH 4.5−5.2) within *Plasmodium falciparum* have revealed a defined metabolic pathway for the degradation of hemoglobin.[2] While hemoglobin proteolysis yields needed amino acids, it also releases toxic free heme (Fe(III)PPIX). To balance the metabolic requirements for amino acids against the toxic effects of heme, malaria parasites have evolved a detoxification mechanism which involves the formation of a crystalline heme aggregate known as hemozoin (malaria pigment, β-hematin).[3] The structure of hemozoin appears to be a coordination polymer of Fe(III)PPIX in which an oxygen from a propionate group from one unit serves as an axial ligand to the five-coordinate ferric ion of another.[4] Recently, Bohle and co-workers have proposed a refined topological model of hemozoin based on X-ray powder diffraction data that includes hydrogen-bonded propionic dimerization between polymer chains.[5]

Within the digestive food vacuole of *P. falciparum*, Goldberg and co-workers have identified a class of histidine-rich proteins (HRP II−IV) containing multiple tripeptide Ala-His-His repeats that mediate the formation of hemozoin and are inhibited by antimalarials such as chloroquine.[6] While there is compelling evidence to suggest a physiological role for the HRPs in the detoxification of heme, the question remains: do these proteins function as enzymes by binding substrate and releasing product, or do they function as nucleating templates for the epitaxial growth of hemozoin? Several researchers have suggested that as both purified hemozoin and synthetic β-hematin can seed

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(2) During the intraerythrocytic phase of its life cycle, the malaria parasite can degrade up to 80% of an infected erythrocyte's hemoglobin to obtain requisite amino acids for its maturation; (a) Olliaro, P. L.; Goldberg, D. E. *Parasitol. Today* **1995**, *11*, 294−297. (b) Rosenthal, P. J.; McKerrow, J. H.; Aikawa, M.; Nagasawa, H.; Leech, J. H. *J. Clin. Invest.* **1988**, *82*, 1560−1566. (c) Goldberg, D. E.; Slater, A. F. G.; Cerami, A.; Henderson, G. B. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 2931−2935.

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the malaria pigment polymerization reaction,[7] the HRPs are more likely to play a role in the nucleation of hemozoin. Furthermore, the presence of the Ala-His-His repeat motif is reminiscent of a variety of nucleating scaffold proteins used in biomineralization.[8] In these systems, the three-dimensional structure of the protein yields a preorganized, functionalized surface that serves as a template for nucleation. To investigate the molecular factors which control hemozoin formation, we have designed and synthesized two peptide dendrimers containing a minimal binding domain sequence to act as templates for the nucleation and growth of hemozoin.

Results and Discussion

Design and Synthesis of Bionucleating Templates. As a result of their characteristic branched structures, peptide dendrimers have found important applications in the development of vaccines,[9] diagnostic products,[10] antibiotics,[11] and protein models.[12] An examination of the Ala-His-His repeats within HRP II reveals that a common organizational element is the tandem 9-mer repeat Ala-His-His-Ala-His-His-Ala-Ala-Asp.[13] Capitalizing on the branched nature of the peptide dendrimer core which allows the presentation of a large number of nucleating domains to solution, we have designed two first-generation bionucleating templates (BNT I and II) to direct the synthesis of hemozoin by coupling the putative nucleating domain of HRP II to a tetralysine dendrimer core (Figure 1). The nucleating domain is the 9-mer N-acylated peptide based on the tandem repeat sequence of HRP II. Synthesis of the nucleating-domain peptide was accomplished using automated standard FMOC solid-phase techniques.[14] The first bionucleating template (BNT I) incorporates four individual binding domains attached to the tetralysine dendrimer core for 8 tri-Ala-His-His repeats, while the second template (BNT II) couples two nucleating domains to each of the branches of the tetralysine core to generate a total of 16 tri-Ala-His-His repeats. The synthesis of BNT I and II was achieved by automated FMOC synthesis protocols for multiantigenic peptides.[15] MALDI-TOF mass spectrometry and HPLC confirmed the authenticity and purity of the synthesized templates (see Supporting Information). Furthermore, BNT I and II tested positive against monoclonal antibodies for HRP II, using a commercial test ("***Para***sight F")[16] for *P. falciparum* malaria, while blank and serum albumin control solutions tested negative.

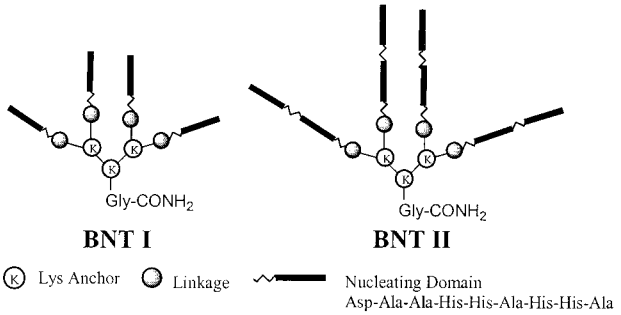

Figure 1. Generalized diagram of the dendrimeric bionucleating templates. For BNT I, there is only one shell of nucleating domains and a single alanine residue linker between the tetralysine core. In BNT II, there are two shells of nucleating domains and no linker.

Table 1. Substrate Binding Stoichiometries of BNT I and BNT II

substrate	BNT I (8)	BNT II (16)
Fe(III)PPIX	7.1 ± 0.7	12.2 ± 1.0
PPIX	7.7 ± 0.2	10.4 ± 1.0
Zn(II)PPIX	6.8 ± 0.5	14.0 ± 0.6
Pcs	5.7 ± 0.2	13.2 ± 1.0
Ni(II)Pcs	6.5 ± 0.1	11.0 ± 1.0

Substrate Binding. The templates were examined for their ability to bind substrates including Fe(III)protoporphyrin IX (Fe(III)PPIX), protoporphyrin IX (PPIX), Zn(II)protoporphyrin IX (Zn(II)PPIX), tetrasulfonatophthalocyanine (PcS), and Ni(II)tetrasulfonatophthalocyanine (Ni(II)PcS) (Table 1). Using difference absorbance methods,[17] Sullivan and co-workers have shown that purified HRP II can bind 17 equiv of Fe(III)PPIX.[6] Under similar conditions, all of the porphyrin and water soluble phthalocyanine substrates were recognized by BNT I and II. Considering the skeletal similarities between porphyrins and phthalocyanines, the recognition of the latter by BNT I and II was not surprising. Assuming each of the Ala-His-His tripeptide repeats corresponds to a substrate recognition site, the examined substrates occupy 75% of the available sites within a given template.[18] Given the geometry of the dendrimeric core, binding sites near the core interior were expected to be less accessible

(7) Recent studies have also examined the possible role of lipids as nucleating templates, showing that an acetonitrile extract of trophozoites and several lipids of undefined phase are also capable of initiating hemozoin formation; (a) Dorn, A.; Stoffel, R.; Matile, H.; Bubendorf, A.; Ridley, R. G. *Nature* **1995**, *374*, 269−271. (b) Dorn, A.; Sudha, R. V.; Matile, H.; Bubendorf, A.; Vennerstrom, J. L.; Ridley, R. G. *Biochem. Pharmacol.* **1998**, *55*, 737−747.

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(13) (a) HRP II (M_r 35 kD) contains 51 repeats of the sequence Ala-His-His (76% of the mature protein is histidine and alanine). HRP III (M_r 27kD) contains 28 Ala-His-His sequences (56% of the mature protein is histidine and alanine). Wellems, T. E.; Howard, R. J. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 6065−6069. (b) HRP IV (M_r 10 kD) contains 31% histidine predominantly. Stahl, H. D.; Crewther, P. E.; Scanlon, D. B.; Woodrow, G.; Brown, G. V.; Bianco, A. F.; Anders, R. F.; Coppel, R. L. *Nucleic Acids Res.* **1985**, *13*, 7837−7846. (c) Lenstra, R.; d′Auriol, L.; Andrieu, B.; Le Bras, J.; Galibert, F. *Biochem. Biophys. Res. Commun.* **1987**, *146*, 368−377.

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(18) Analysis of the binding data to determine K_D is complicated by the variety of aggregation processes ongoing at the experimental pH. Future equilibrium binding studies are planned in order to provide better estimates of the apparent substrate K_D.

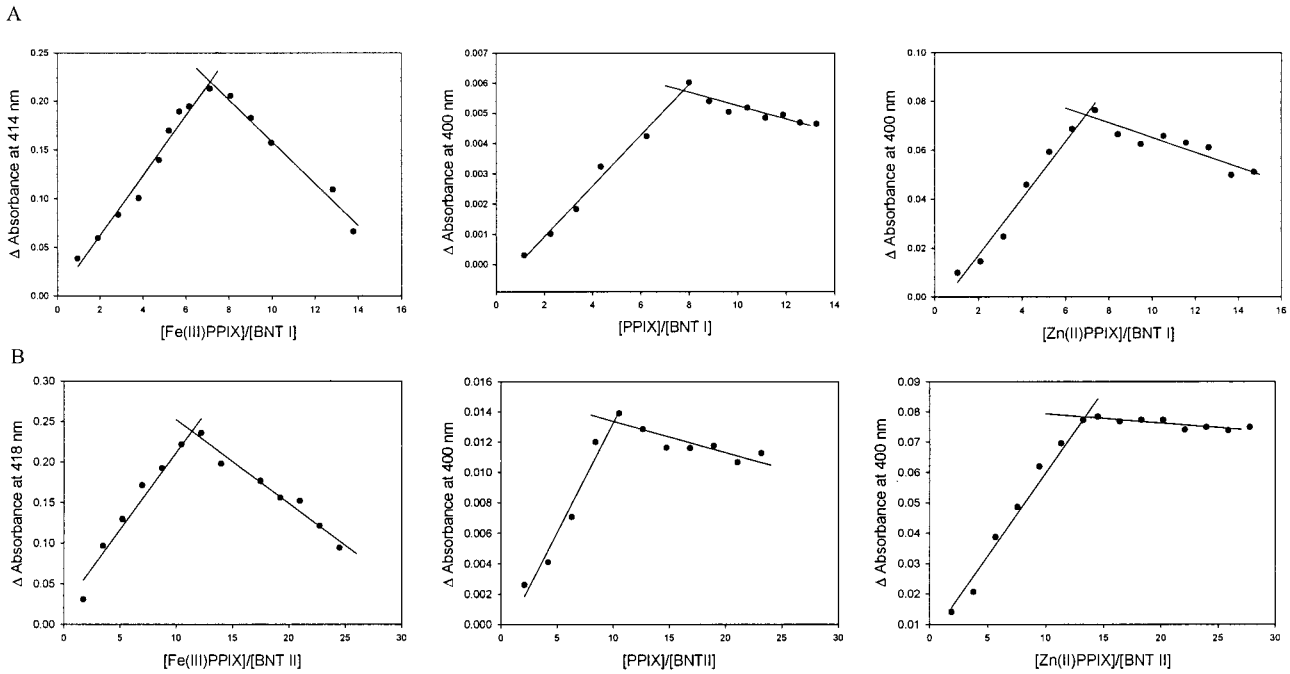

Figure 2. Substrate binding titrations for (A) BNT I and (B) BNT II with Fe(III)PPIX, PPIX, and Zn(II)PPIX substrates. Binding studies were based on the UV−vis difference titration methods of Morgan.[17] Aliquots of a stock solution of substrate (1 mM) dissolved in 0.1 M NaOH were added to a 100 mM acetate buffer solution (pH 4.8) containing the appropriate amount of nucleating template (1−1.5 μM) and a 100 mM acetate buffer solution blank.

than those closer to the surface,[19] resulting in the lower substrate-loading stoichiometries of BNT I and II.

The fact that protoporphyrin IX (PPIX) was capable of binding to the nucleating templates suggests that association of the substrate with template is not driven by axial ligation of a histidine residue to a metal ion.[20] Given that the pK_a of the imidazole side chain of histidine is 5.8, rendering the moiety protonated under the examined reaction conditions, axial ligation to a metal is expected to be unlikely. These results suggest that binding of the substrate is more likely mediated via a combination of π-stacking[22] and electrostatic interactions. Association of the metalloporphyrin substrate with the binding site via noncovalent interactions distinguishes metalloporphyrin binding to the histidine-rich proteins of *P. falciparum* from that of the histidine proline-rich glycoproteins found in many mammals. The later demonstrate axial bis-histidine ligation of heme substrates at pH 7.4 within a histidine-rich binding domain.[17,21]

After the stoichiometric binding maxima, the difference titration curves for the Fe(III)PPIX substrate reflect a significant change in the electronic structure of the species in solution (Figure 2 and Supporting Information). In contrast, the titration curves of the other substrates show only a slight negative divergence from the plateau expected with saturable binding of the template. At high stoichiometries of substrates, a number of aggregative interactions are possible which eventually result in precipitation. Such interactions are likely responsible for the slight negative trend noted for PPIX and Zn(II)PPIX after maximal binding. Although there are several possible interpretations for the observed spectral behavior of the Fe(III)PPIX/template solution, the most intriguing is that the data represent the onset of Fe(III)PPIX aggregation with a heme/template complex[22] in a nucleation step that is a prelude to hemozoin formation.

Heme Polymerization Activity. The templates were analyzed for their ability to produce hemozoin using the *in vitro* heme polymerization assay of Sullivan and co-workers.[6] In the presence of free heme under acidic conditions, the templates promoted the formation of insoluble aggregated heme products, whereas proteins such as bovine serum albumin or the amino acid polymer polyhistidine did not (Figure 3). The differential solubility of the isolated material was carefully examined to optimize phase homogeneity by an exhaustive washing cycle of bicarbonate buffer (pH 9.5), DMSO, and water.[23] The amount of insoluble aggregated heme derivatives formed was dependent on the number of binding domains linked to the tetralysine core (Figure 3), the template concentration, and the incubation period of the reaction (Figure 4a). The templates had an optimal pH regime between 4.0 and 4.5 (Figure 4b). As the pH of the assay approached the pK_a of the imidazole side chain of the histidine residues, the polymerization activity was dramatically curtailed. Furthermore, chloroquine inhibited the template-mediated synthesis of hemozoin-like material, consistent with previous reports of HRP II inhibition by this drug (Figure 5a). Fourier transform infrared spectroscopy of the template reaction product exhibited the characteristic vibrational frequencies of hemozoin at 1660 and 1210 cm^{-1} (Supporting Information).[4b,6,24] While the synthetic templates produced yields on the same order of

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(20) Further, UV−vis of the template-associated Fe(III)PPIX complex does not show any of the features associated with histidine axial ligation (Supporting Information).

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(23) Olliaro, P. Differential solubility is a common bench test for the presence of hemozoin. Free heme is soluble in the sodium bicarbonate buffer (pH 9.5), while hemozoin is not. Recent studies have suggested that a DMSO wash removes short oligomers from the bulk polymer. Personal communication.

(24) Bürger, H. In *Porphyrins and Metalloporphyrins*; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; 115.

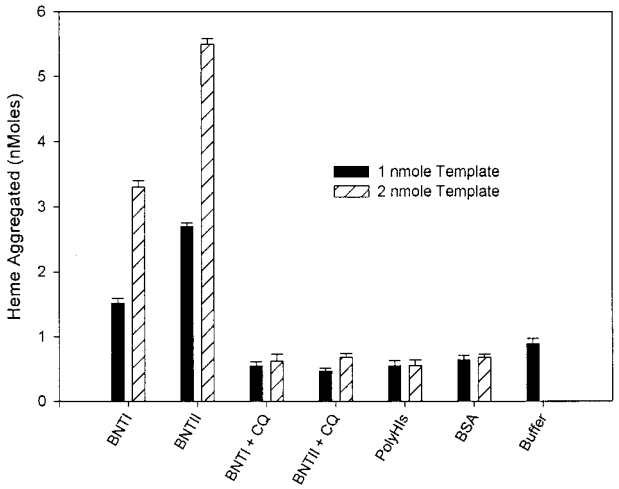

Figure 3. Hemozoin production mediated by bionucleating templates. Representative polymerization assay with 50 μM of hemin in 2 mL of acetate buffer (500 mM, pH 4.8) at 37 °C. BNT I and BNT II were used in 1 and 2 nmol amounts. Chloroquine (CQ, 100 μM) was included with BNT I and BNT II in inhibition reactions. Polyhistidine and bovine serum albumin in approximately 1 and 2 nmol amounts were used in protein-control experiments. The blank control was the acetate buffer above. Baseline amounts of insoluble aggregate are consistent with those previously reported under similar conditions.[7b]

magnitude as HRP II, the rate at which hemozoin is formed by BNT I and BNT II is approximately 15% and 30% that of the protein, respectively.

Previous studies have shown that linear multimers of the hexapeptide Ala-His-His-Ala-Ala-Asp bind stoichiometric amounts of heme, but do not promote the polymerization of hemozoin.[25] Similarly, the amino acid polymer polyhistidine binds heme but does not yield hemozoin. In contrast, the tandem repeats which form the binding domains of the dendrimeric bionucleating templates I and II not only bind heme substrate but form hemozoin. The difference in observed behaviors between nonproductive and productive peptide templates is illustrative. Geometric and steric constraints inherent to the dendrimeric scaffold, which the linear multimers do not contain, limit possible nonproductive bis-heme associations (both covalent or noncovalent inter- and intrapeptide). Previous work on the linear heme binding peptide from the histidine proline-rich glycoprotein of mammals has demonstrated that the sequence Gly-His-His-Pro-His-Gly provides a bis-axial histidine coordination environment in model studies.[17] Such interactions[17,21] would result in a "capped" heme unable to propagate the hemozoin coordination polymer. With such interactions attenuated, the bionucleating templates display a higher effective concentration of nucleating domains to bind substrate. The activity differences noted for polyhistidine and the bionucleating templates suggest that the sequence of the nucleation site is, in fact, important for hemozoin initiation. Current results suggest a model for hemozoin nucleation based upon the occupation of tandem Ala-His-His sites within HRP by heme substrate.[26] Such a model would provide the appropriate spatial disposition of histidine functional groups along the template's surface to

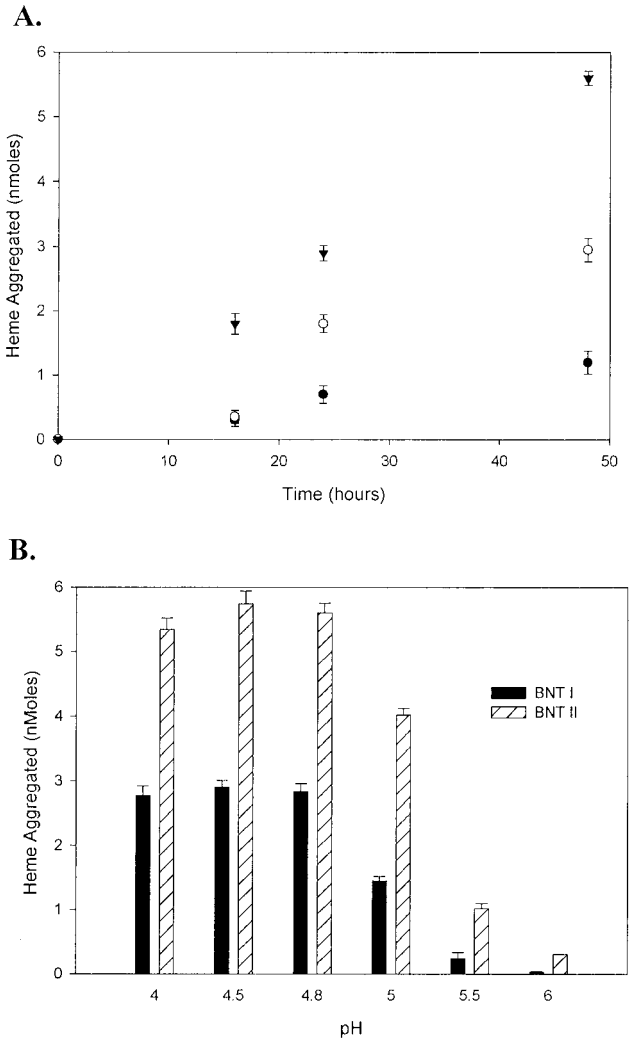

Figure 4. (A) Time evolution for the bionucleating template mediated production of insoluble heme aggregates: ●, blank; ○, BNT I; ▼, BNT II. Hemin (50 μM) in 2 mL acetate buffer (500 mM, pH 4.8) at 37 °C was incubated with 2 nmol of BNT I or BNT II for the indicated intervals. (B) pH dependence of the bionucleating template mediated formation of insoluble heme aggregates. Hemin (50 μM) in 2 mL acetate buffer (500 mM, pH 4.8) at 37 °C was incubated with 2 nmol of BNT I or BNT II at the indicated pH for 48 h.

generate not only sites of substrate recognition but also the longer range translational symmetry of the crystalline polymer lattice.[27] An examination of HRP II protein sequence[13] reveals that of the 51 Ala-His-His repeats, 32 of them are organized into 16 tandem nucleating domain motifs. Indiscriminate heme substrate interactions along polyhistidine do not allow for such organization.

Recently, it has been demonstrated that Zn(II)PPIX and related analogues inhibit in vitro hemozoin formation in *P. falciparum* trophozoite extracts.[28] It has been hypothesized that the increased levels of Zn(II)PPIX found in thalassemic erythrocytes may contribute to the observed antimalarial protection conferred by the β-thalassemia trait. While the causative

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(26) It should be emphasized that the nucleation model of Ala-His-His-Ala-His-His-Ala-Ala-Asp presented above does not represent a unique organization of the Ala-His-His tripeptides in HRP II, but simply an initial organization which results in nucleation. An alternate sequence currently under investigation is Ala-His-His-Ala-Ala-Asp-Ala-His-His. Tandem occupation of each Ala-His-His subsite would still be required for hemozoin nucleation.

(27) Such a function has been attributed to many scaffolding proteins. Relevant examples include (a) the α-helical anti-freeze protein of fish; Madura, J. D.; Wierzbicki, A.; Harrington, J. P.; Raymond, J. A.; Sikes, C. S. *J. Am. Chem. Soc.* **1994**, *116*, 417. (b) the β-pleated sheet proteins of many sea organisms; Albeck, S.; Aizenberg, J.; Weiner, S. *J. Am. Chem. Soc.* **1993**, *115*, 11691 and Aizenberg, J.; Hanson, J.; Koetzle, T. F.; Weiner, S.; Addadi, L. *J. Am. Chem. Soc.* **1997**, *119*, 881.

(28) Martiney, J. A.; Cerami, A.; Salter, A. F. G. *Mol. Med. (Tokyo)* **1996**, *2*, 236−246.

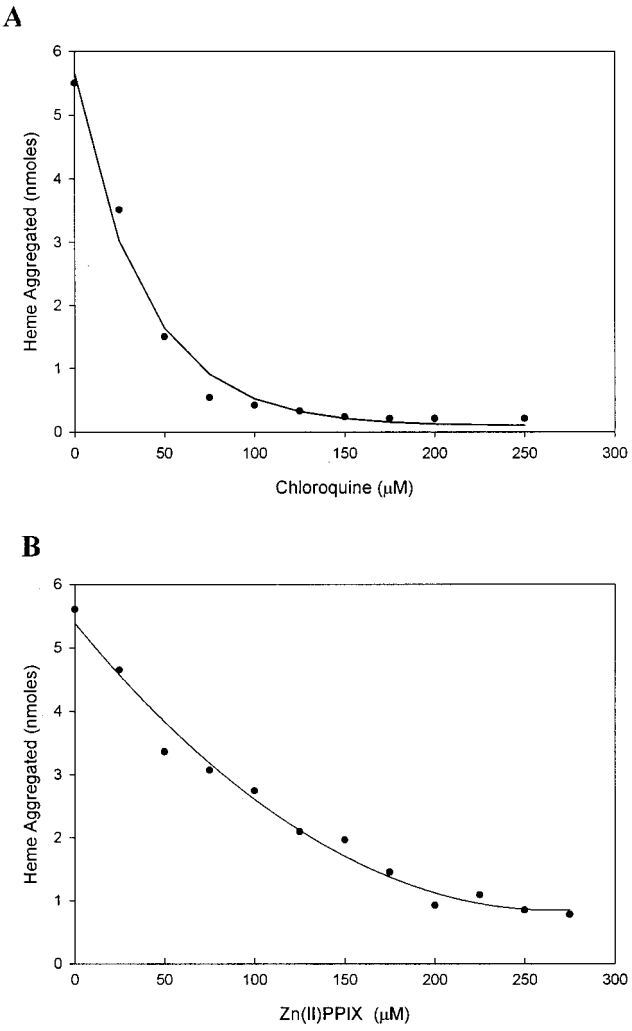

Figure 5. Inhibition of bionucleating template mediated hemozoin production. (A) Effect of increasing concentrations of the known antimalarial chloroquine. Results of the assays consisting of 50 μM of hemin and 1 μM BNT II in 2 mL of acetate buffer (500 mM, pH 4.8, 37 °C) and increasing concentrations of chloroquine reveal an $IC_{50} \approx 35$ μM. Each point represents the average of three individual in vitro assays with a standard deviation of no more than 5%. (B) Effect of increasing concentrations of Zn(II)protoporphyrin IX. Results of the assays consisting of 50 μM of hemin and 1 μM of BNT II in 2 mL of acetate buffer (500 mM, pH 4.8, 37 °C) and increasing amounts of Zn(II)PPIX reveal an $IC_{50} \approx 125$ μM. Each point represents the average of three individual in vitro assays with a standard deviation of no more than 5%. The line through the data represents a multiorder exponential fit.

agent of hemozoin formation in these extracts was not identified, HRP II has been shown to be present during the trophozoite stage within the digestive vacuole and mediate hemozoin formation.[6] Binding of Zn(II)PPIX to the template models of HRP II (Figure 2) suggests a possible mode for the inhibition of heme polymerization. Namely, the nucleation sites within the histidine-rich protein are occupied by Zn(II)PPIX, thereby preventing nucleation of Fe(III)PPIX. The concentration dependence of Zn(II)PPIX on the inhibition of template mediated hemozoin formation is shown in Figure 5. The half-maximal inhibitory effect (IC_{50}) of Zn(II)PPIX is observed at a concentration of 125 μM. Further, the differences between the IC_{50} of chloroquine and that of Zn(II)PPIX reported here are similar to results previously obtained for trophozoite extract-derived heme polymerization activity.[28] The fact that a large number of porphyrin and related macrocyclic complexes can bind the HRP II nucleating domain highlights the potential for these complexes as novel platforms from which to probe aspects of hemozoin formation.

We have examined two peptide dendrimers based on the tandem repeat motif of HRP II from *P. falciparum* for their abilities both to bind heme substrates and to form the critical detoxification polymer hemozoin. Each template is capable of binding significant amounts of the natural substrate, Fe(III)PPIX. Binding of the metal-free base protoporphyrin IX to the templates suggests, however, that substrate recognition is based on the porphyrin moiety rather than specific-metal recognition. Such a supposition is further supported by the fact that Zn(II)PPIX, as well as the structurally related metal-free and metallo phthalocyanines, can bind to the templates. Further, it has been shown that the dendrimeric BNT I and BNT II are capable of supporting the polymerization of hemozoin. In light of previously reported binding studies of linear sequences derived from HRP II and the polymer polyhistidine, the results suggest that the tandem organization of the tripeptide binding sites promotes the formation of hemozoin for these model templates. Ongoing studies of these synthetically accessible, biologically active peptide dendrimers are expected to provide additional details concerning both the nature of heme binding to the template and the mechanism of aggregate growth.

Experimental Section

Reagents. All resins and Fmoc amino acids from Perseptive Biosystems (Framingham, MA) and Fmoc-Lys(Fmoc)-OH (NovaBiochem, San Diego, CA) were used as received. HBTU and HOBT were purchased from Advance ChemTech (Louisville, KY). Porphyrins were purchased from Porphyrin Products (Logan, UT). Phthalocyanines were purchased from Fluka. All other reagents were obtained from Fisher Scientific (Pittsburgh, PA). All solvents were HPLC grade or of equivalent purity.

Synthesis of Bionucleating Templates. The dendrimeric templates were synthesized using standard continuous-flow Fmoc solid-phase peptide-synthesis methods on a PerSeptive Biosystem Pioneer peptide-synthesis system[14,15] on PAL-resin. All coupling cycles were carried out with a sixteen-fold excess (vs load capacity of resin) of activated amino acid, using Fmoc amino acid/HBTU/HOBt/DIEA (1:1:1:2) in DMF for a period of 1.5 h. Fmoc-Lys(Fmoc)-OH was used to obtain the branched peptide. Fmoc groups were removed by treatment with 20% piperidine in DMF. Acylation of the N-terminal amines was performed with the exposure of the peptidyl resin to acetic anhydride/pyridine/DMF (1:1:5) for 15 min. The branched peptides were cleaved by mixing 1 g of dried resin with 95% TFA/55% TIS (10 mL) for 2 h at room temperature, were filtered, and were washed with 95% TFA (10 mL). The peptide was precipitated with ether, redissolved in a minimum volume of 20% CH_3CN in water, and lyophilized. Peptides were purified by reversed-phase HPLC with a Rainin HPXL system equipped with a Dynamax Model UV-1 variable-wavelength detector and Rheodyne injector using a Microsorb C_{18} reversed-phase column (Rainin, 21.4 × 250 mm), variable back pressure (1600−1900 psi), and a flow rate of 21 mL/min at 25 °C.

Porphyrin Binding Assay. Binding studies were based on the UV−vis difference titration methods of Morgan.[17] Aliquots of a stock solution of substrate (≈ 1 mM) dissolved in 0.1 M NaOH were added to a 100 mM acetate buffer solution (pH 4.8) containing the appropriate amount of nucleating template (10−110 nmol) and a 100 mM acetate buffer solution blank. Samples were equilibrated for 15 min prior to measurements.

In Vitro Heme Polymerization Assay. The bionucleating templates were assayed for hemozoin formation by the incubation of 1−2 nmol of template with 50−100 μM of hemin in 2 mL of a 500 mM sodium acetate buffer (pH 4.8) for 16, 24, and 48 h as previously described. After centrifugation at 15 000 g for 1 h, the pellet was resuspended in 0.1 M sodium bicarbonate (pH 9.1) to solubilize free heme. The pellet was sonicated, vortexed, and centrifuged for 1 h. The supernatant was

discarded. The remaining pellet was washed with ddH_2O, sonicated, vortexed, and centrifuged. Samples were quantitated on the basis of absorbance at 400 nm in a 1 mL 0.1 M NaOH solution.

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Supporting Information Available: Summary table of peptide and peptide dendrimer synthesis. HPLC traces and MALDI-TOF mass spectrometry for the nucleating domain peptide and BNT I and BNT II. UV−vis of Fe(III)PPIX bound to the nucleating domain peptide, BNT I and BNT 2. UV−vis difference titrations of substrate binding for BNT I and BNT II. FT-IR of template heme aggregate products. This material is available free of charge via the Internet at http://pubs.acs.org.

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