Heme binding and polymerization by *Plasmodium falciparum* histidine rich protein II: influence of pH on activity and conformation

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Abstract The histidine rich protein II (HRPII) from *Plasmodium falciparum* has been implicated as a heme polymerase which detoxifies free heme by its polymerization to inactive hemozoin. Histidine-iron center coordination is the dominant mechanism of interaction between the amino acid and heme. The protein also contains aspartate allowing for ionic/coordination interactions between the carboxylate side chain and the heme metal center. The pH profile of heme binding and polymerization shows the possibility of these two types of binding sites being differentiated by pH. Circular dichroism studies of the protein show that pH and heme binding cause a change in conformation above pH 6 implying the involvement of His-His+ transitions. Heme binding at pHs above 6 perturbs HRPII conformation, causing an increase in helicity.

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1. Introduction

Plasmodium sp., the causative agent of malaria, during the trophozoite blood stage of its life cycle, degrades erythrocyte hemoglobin as an essential source of nutrients [1]. The free heme released as a by-product is toxic, inhibiting vacuolar processes and damaging cell membranes.

In the host, the enzyme heme oxygenase detoxifies free heme into biliverdin, which in turn is converted by biliverdin reductase to, and excreted as, water-soluble bilirubin [2]. In the parasite, which lacks heme oxygenase, detoxification is by polymerization of free heme into inert hemozoin [3]. Hemozoin is a compound structurally similar to β-hematin where ionic bonds link the propionate side chain of one hematin moiety to the ferric iron atom at the center of the adjacent hematin moiety [4]. This unique mechanism of heme detoxification by Plasmodia is thus a high-priority target for antiparasitic drugs [5]. The antimalarial activity of chloroquine [6–8] and other antimalarial quinolines [9] is believed to be through the inhibition of this process.

The mechanism of heme polymerization is being debated [10]. Dorn et al. [11] have shown that the chemical environment of the food vacuole, where hemoglobin degradation and the formation of hemozoin occur, is favorable to heme polymerization. Specifically the pH of 4.8–5.3 encourages ionic polymerization by both shifting the equilibrium of heme from the μ-oxo dimer state to the monomer [10], and allowing for ionization of the propionic side chains (pK = 5.0 [12]) of heme. They have shown the ability of hemozoin itself to autocatalyze additional hemozoin [13]. A non-protein acetonitrile extract of the trophozoite [10] and non-physiological lipids [14] also catalyze hemozoin formation, questioning the need for a 'heme polymerase' enzyme.

Recent candidates identified as heme polymerases are the histidine rich proteins HRPII and HRPIII [15]. These proteins were shown to be present in the food vacuole, and to unambiguously polymerize heme [15]. Synthetic peptides corresponding to a repetitive sequence of HRPII bind heme and inhibit hemozoin formation in vitro [16]. The similarity of the repetitive sequence Ala-His-His-Ala-Ala-Asp, to the heme binding site of histidine rich glycoprotein (HRG), Gly-His-His-Pro-His-Gly, was also supportive of the hypothesis that HRPII polymerizes heme [15].

However, in HRG (and most proteins involved in heme binding with histidine), binding takes place between histidine and the heme iron center [17]. The histidine side chain has a pK value of 6.0, and histidine-iron association is only possible above this value. HRPII, being active as a catalyst in the food vacuole, which has a pH of 4.8–5.3 – below the pK_a of histidine – must therefore allow for a mode of binding very different from the norm. HRPII is largely made up of repeats involving three amino acids: histidine (34%), alanine (37%) and aspartic acid (10%) [18]. The high proportion of aspartic acid is suggestive of the role of this amino acid in heme binding at pHs less than 6.0. To probe this hypothesis and to understand the nature of heme polymerization by HRPII and HRPIII, we report the variation of recombinant HRPII activity and conformation with pH and heme.

2. Materials and methods

2.1. Construction of the HRPII expression vector

Details of the cloning, expression and purification of HRPII will be published elsewhere. Briefly, the gene encoding HRPII was cloned from the genomic DNA of *P. falciparum* by polymerase chain reaction (PCR). The sequences of the primers (sense, 5′-CCGGAATTCATGAATAATTCCGCATTTAAT and antisense, 5′-GCCGACGTCGACTTAATGGCGTAGGCAATG-3′) were designed on the basis of reported DNA sequences of HRPII [18] so as to introduce an *Eco*RI site at the N-terminus of the open reading frame and a *Sal*I site just after the C-terminus. The 0.9 kb PCR product was digested with *Eco*RI and *Sal*I and cloned into an expression vector developed in house [19]. This vector has a strong tightly regulated promoter of bacteriophage lambda (PL) which is thermoregulated due to the presence of a heat labile c 1857 repressor. The plasmid was transformed into *Escherichia coli* strain BL21 and the sequence was verified using a dideoxy sequence method.

2.2. Purification of HRPII

E. coli BL21 cells harboring the plasmid encoding HRPII were cultured at 29°C overnight in 5 ml of LB medium containing

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Abbreviations: HRPII and HRPIII, histidine rich proteins II and III; HRG, histidine rich glycoprotein; CD, circular dichroism; PCR, polymerase chain reaction

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100 μg/ml ampicillin and were then transferred to 2 l of the same medium. The cells were grown for 4 h at 29°C. When the OD_{600} reached 0.6, the flask was shaken in a water bath at 70°C for 2 min. The cell growth was continued at 42°C for 4 h. Cells were harvested, and disrupted to release the protein using sonication. After centrifugation, the supernatant was loaded onto a 5 ml column of Ni^{2+} NTA-agarose equilibrated with buffer. HRPII was eluted by an imidazole gradient from 200 to 1000 mM. The HRPII protein was eluted at about 300 mM imidazole and was dialyzed against different buffers for analysis.

2.3. Estimation of heme binding

Stock solutions of hemin (Sigma Chemical Co.) were freshly prepared by dissolving it in a minimal quantity of 0.1 M NaOH, and filtering. The concentration of the stock was determined by diluting 1000-fold in DMSO and measuring the absorbance at 405 nm ($\varepsilon_{405} = 170\,000$ [20]). Forty equivalents of heme was added to a 10 ml sample containing 10 nM HRPII in 10 mM sodium phosphate buffer, pH 8. The protein concentration was determined using the BCA protein assay (Pierce Chemical Co.). The pH was lowered with hydrochloric acid to the indicated values and the absorption spectrum of each from 450 to 325 nm was recorded. Volume changes were less than 5%. The same procedure was followed for heme in phosphate buffer, as a control.

2.4. Hemozoin estimation

Samples of the protein were prepared to a final concentration of 10 pmol in either 100 mM acetate or phosphate buffer depending on the required pH. 50 nmol of hemin was added to the sample tube from a freshly prepared stock. Each assay was set up in triplicate and incubated at 37°C overnight, on a rotary shaker. The reaction was stopped by adding 10 μl of 10% SDS, vortexing and then centrifuging the sample at $16\,000 \times g$ for 1 h at 25°C. The pellets were resuspended in 2.5% SDS and sodium bicarbonate solution (100 mM, pH 9.0), sonicated at low power for 10 s and the pellet was again collected by centrifugation. This step dissolves most of the free heme attached to the hemozoin and other non-hemozoin-like adducts which form spontaneously during the assay. The pellet thus obtained is of polymerized heme.

For heme quantitation the pellets were solubilized in 20 mM NaOH to convert polymerized heme (hemozoin/β-hematin) to hematin and absorbance was measured at 400 nm.

2.5. Circular dichroism (CD) measurements

CD spectra were recorded in the far UV range from 260 nm to 190 nm, on a Jasco J720 spectropolarimeter with an attached data processor. CD spectra were acquired for samples prepared in the buffer used. The spectra of the buffer alone and with heme were later subtracted from the recorded spectra. Typical parameters used in recording the spectra were: bandwidth 1 nm, response time 2 s, and scan speed 100 nm/s, with spectral averaging over five accumulations. Data were analyzed using non-linear curve fitting with a logistic function from Sigmaplot (Jandel Scientific).

3. Results and discussion

3.1. Effect of pH on binding and polymerization of heme

From the pH profile of HRPII-heme interactions (Fig. 1), a decrease in the binding is seen below pH 7.3, with a mid-point around pH 6.5. Other heme binding proteins (e.g. HRG [20] and hemoplexin [21]) involving histidine-iron interactions show markedly similar behavior. This rapid fall in binding is attributed to the change in the charge of the side chain from His to His+ ($pK = 6.0$), the positively charged side chain being unable to bind the metal. In HRG, heme binding reduces to baseline levels as only histidine-heme binding is involved, with histidine having an apparent pK_a of 5.8 [20]. However, HRPII, in comparison to HRG, shows some residual binding of heme below pH 6, 3–4-fold lower than binding at higher pH (Fig. 1). This indicates the possibility of a second type of binding site, which can be differentiated by pH.

Hemozoin formation by HRPII shows a pattern inversely related to the binding (Fig. 1), being optimum around pH 5 and reaching baseline levels rapidly above pH 6. This pH dependent pattern was suggested in an earlier report that defined HRPII involvement as a heme polymerase [15] and is comparable with recent extensive studies on hemozoin formation [11]. Fig. 1 indicates that hemozoin formation is not associated with the histidine-iron binding, but with the second type of binding as suggested by the pH profile of binding.

Metal binding properties are associated with amino acids containing carboxylate, imidazole and hydroxyl side chains [22]. The variation of heme binding with pH is consistent with the model that both histidine and aspartate based interactions with heme are involved. Based on the classical pK_a of these amino acids, binding below pH 6 would be aspartate carboxylate-metal ionic/coordinate interactions and the increased binding above pH 6 from histidine-metal coordination. The chemical conditions of the food vacuole, where

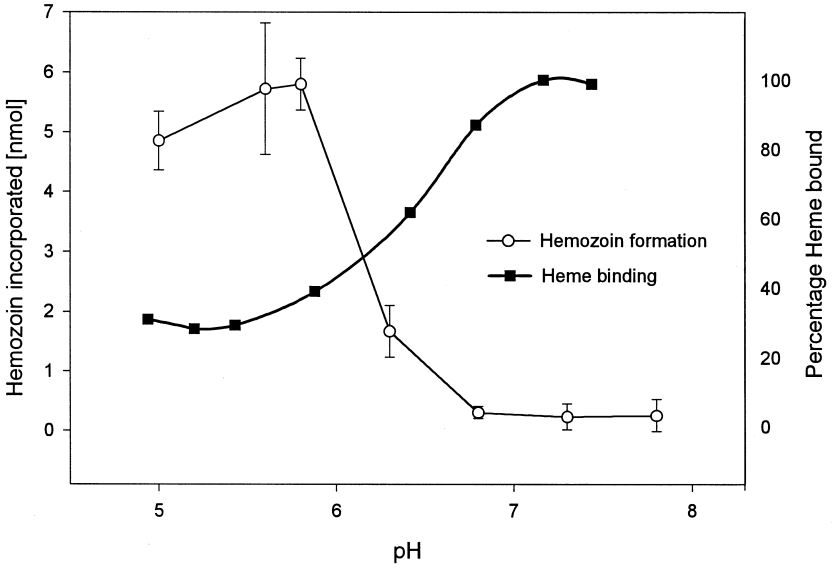

Fig. 1. HRPII binding and polymerization of heme: variation with pH.

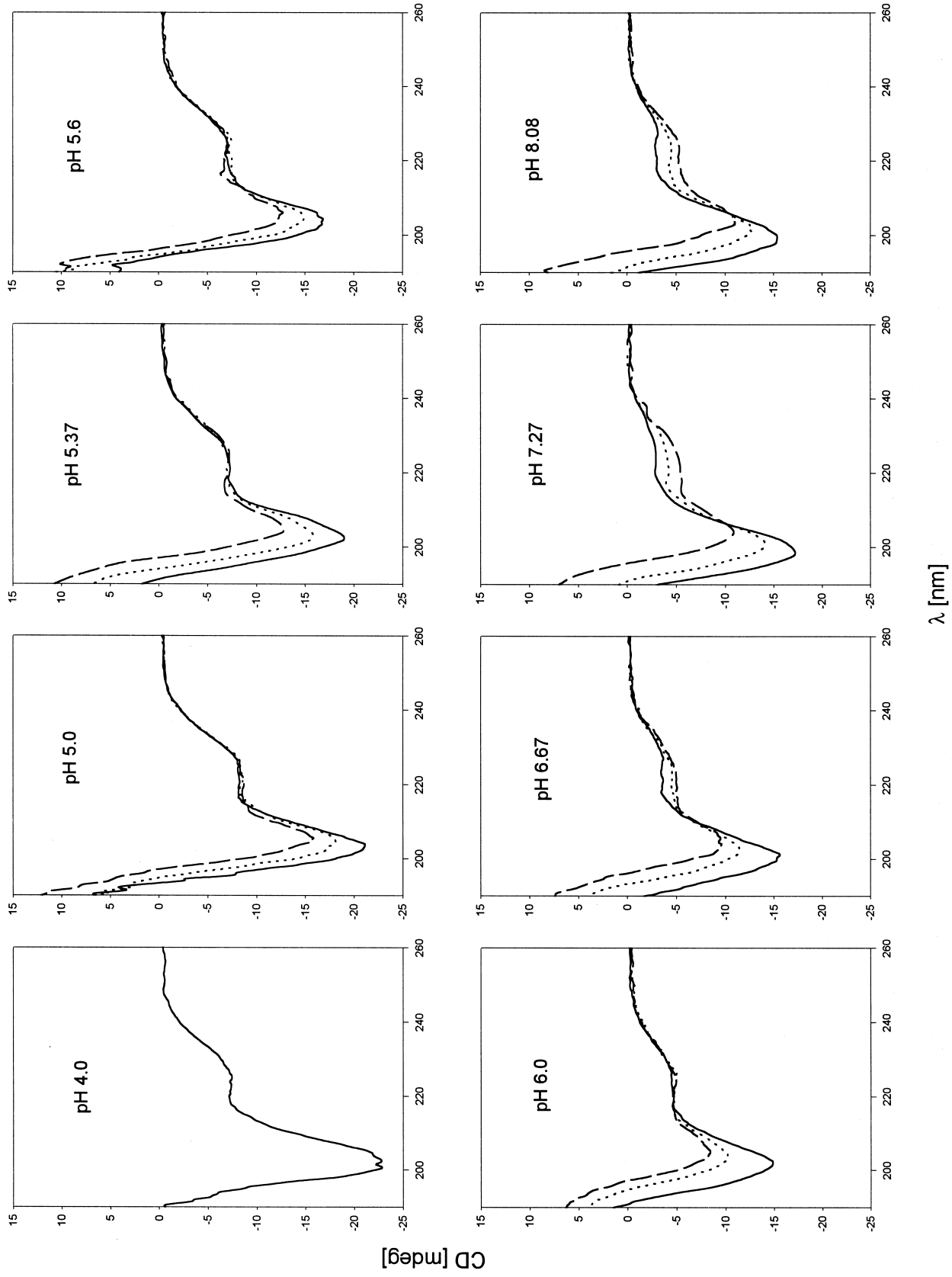

Fig. 2. CD spectra of HRPII. Effect of heme binding and pH. The figure shows HRPII in buffer (solid line) and with 10 (dotted line) and 40 (dashed line) equivalents of heme.

heme polymerization takes place, do not affect the charged state of the aspartate side chain ($pK_a = 4.4$).

3.2. Conformational changes with pH and heme

The CD spectrum of rHRPII (Fig. 2) shows a deep negative peak at 200 nm and a shoulder of varying depth around 220 nm. The spectrum is similar in nature to published spectra of native HRPII [23]. The negative peak at 200 nm is normally taken as a signature of random-coil/unordered structure in polypeptides. A similar curve is shown by polyproline in TFE, where it forms a left-handed helix with three residues per turn (denoted a poly pro II or PP II conformation) and occurs to a significant extent in globular proteins [24]. Anomalous movement on SDS, stability over a broad range of pHs and temperatures is, however, indicative of a highly ordered conformation in this protein. A good reference CD spectrum of a 3_{10} helix is not available owing to the low frequency of occurrence of this protein, preventing the unambiguous definition of HRPII conformation from CD data alone.

The negative ellipticity at 222 nm is a recognized marker of α-helicity in polypeptides, contributions from other secondary structures at this wavelength being negligible [25,26]. This parameter is useful to monitor the effect of pH and heme binding on the conformation of HRPII. The pH profile of this parameter with HRPII shows that there are two levels of helicity below and above pH 6 (Fig. 3). With heme, below pH 6, there is no change in helicity, but above this pH, increasing additions of heme to the sample cause a corresponding increase in helicity (Fig. 4).

p*K* values of histidine vary with sequence and chemical environment [27]. These fluctuations around pH 6 seen in Figs. 3 and 4 are additional indicators of the involvement of the His-His+ transition in HRPII-heme interactions, and that

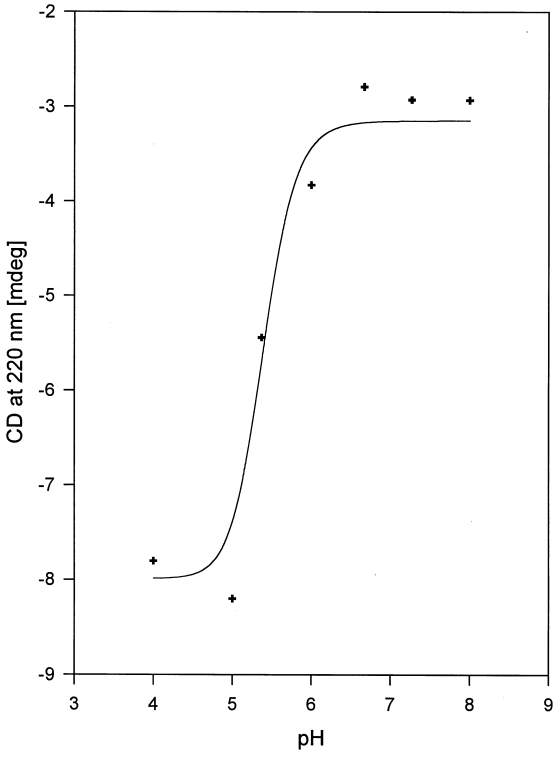

Fig. 3. Effect of pH on the helicity of rHRPII.

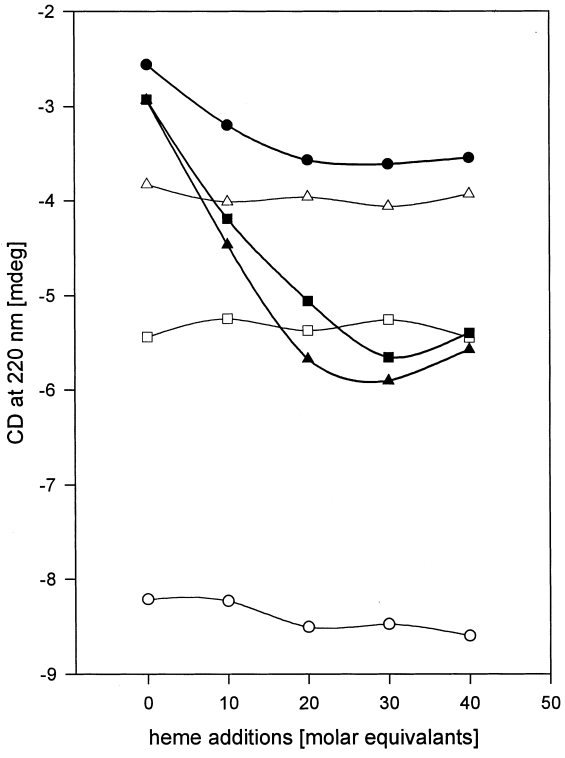

Fig. 4. Effect of heme binding on helicity of HRPII at different pH. ○, pH 5; □, pH 5.6; △, pH 6.0; ●, pH 6.67; ■, pH 7.27; ▲, pH 8.08.

the bulk of the histidine residues maintain p*K* values close to the classical p*K* values. HRPII is largely made up of repeats of the sequences AHHAAD and AHHAHHAAD [18]. In a 3_{10} helical conformation, the molecule would be amphipathic with all the alanine residues aligned on one face and the charged residues on the opposite face. The amphipathic nature of the structure would be lost in an α-helical model. This model would, thus, be intrinsically unstable as it would involve the exposure of apolar alanine to the solvent. His+ salt bridges with the negatively charged Asp may stabilize the increased α-helical content at low pH. The absence of these salt bridges due to the changed charge state of the His side chain above pH 6 may explain its reduced α-helical content. Histidine is, however, capable of binding heme at these pHs. The perturbation caused by binding of bulky heme to adjacent histidine residues, and the exclusion of solvent molecules by a layering of heme around the protein surface, may stabilize the increased α-helical content above pH 6, seen in Fig. 4. Heme causes no change to the protein structure below pH 6. Binding to aspartate, assumed to be the dominant form of binding at these pHs, would not cause the same perturbations to conformation as the aspartate residues are spaced 5–8 amino acids apart.

4. Conclusion

The HRPII-heme binding data are suggestive of the two binding sites possible from the sequence. Based on classical p*K* values of histidine and aspartate, binding below pH 6 is assigned to carboxylate-metal ionic interactions involving aspartate and the increase in binding above pH 6.0 is attributed

to an imidazole-iron center type of binding involving histidine. Hemozoin formation correlates with aspartate binding and not with histidine binding. On varying pH, a structural transition is seen around pH 6, consistent with this model. Binding of heme above pH 6 causes a perturbation in the conformation with an increase in the helicity of the protein.

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