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Heme-binding properties of heme detoxification protein from *Plasmodium falciparum*

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ABSTRACT

The heme detoxification protein of the malaria parasite *Plasmodium falciparum* is involved in the formation of hemozoin, an insoluble crystalline form of heme. Although the disruption of hemozoin formation is the most widely used strategy for controlling the malaria parasite, the heme-binding properties of heme detoxification protein are poorly characterized. In this study, we established a method for the expression and purification of the non-tagged protein and characterized heme-binding properties. The spectroscopic features of non-tagged protein differ from those of the His-tagged protein, suggesting that the artificial tag interferes with the properties of the recombinant protein. The purified recombinant non-tagged heme detoxification protein had two heme-binding sites and exhibited a spectrum typical of heme proteins. A mechanism for hemozoin formation is proposed.

1. Introduction

Malaria infection affects hundreds of millions of people worldwide annually, inflicting high levels of mortality. The malaria parasite invades and then replicates within red blood cells. During growth, the parasite obtains nutrients by digesting hemoglobin within a food vacuole [1,2]. This process releases free heme, which is extremely toxic to the parasite [3–5]. While free heme is detoxified by enzymatic degradation in mammals, the heme degradation mechanism of the parasite has not been identified [6]. To sustain growth, the parasite converts free heme into an insoluble crystalline form called hemozoin (Hz) that is not toxic to the parasite [7,8]. Crystal structure analysis reveals that Hz consists of a cyclic dimer of heme in which an iron-oxygen coordinate bond links the central iron of one heme to the oxygen of the propionate group of the adjacent heme [9]. The molecular mechanisms of the Hz formation are critical for the proliferation of malaria parasites; currently used anti-malarial drugs, such as chloroquine and mefloquine, are thought to kill the parasites by inhibiting Hz formation [10,11]. These drugs are thought to impede the formation of Hz by directly binding to free heme, although the actual mechanism of inhibition of Hz production has not been defined. The process remains one of the most promising targets for the development of anti-malarial drugs, because highly specific inhibition of the Hz formation is required for drug activity.

Although several mechanisms have been proposed for the production of Hz, the molecular mechanism is highly controversial. Lipids, histidine-rich proteins, or a combination of the two have been proposed to catalyze the formation of Hz [12–14]. Recently, heme detoxification protein (HDP), which converts heme into Hz, was identified in the malaria parasite [15–17]. The activity of Hz production by HDP is extremely high compared to other known catalysts [15]. HDP and falcipain-2, a principal hemoglobinase, are present in a ~200-kDa protein complex within the food vacuole that produces Hz [18]. Although the presence of two or three heme-binding sites in HDP and the rapid rate of conversion suggest that HDP is involved in the formation of Hz [15], the detailed molecular mechanism of Hz formation has not been revealed. Since HDP has no homology to any known heme-binding proteins [15], the structural and functional characterization of HDP is crucial for understanding Hz formation. In a previous study, recombinant histidine-tagged HDP protein was purified from crude extracts [15]. However, since the histidine tag may interact with heme iron [19,20], we performed expression and purification of non-tagged HDP in *Escherichia coli*. As we report here, comparison of non-tagged HDP with His-tagged HDP revealed that the purification tag affected the heme coordination structure, suggesting that the use of a non-tagged HDP is important for characterizing HDP in its "native" state. We here show that the non-tagged HDP exhibited a spectrum typical for heme proteins and had two heme-binding sites.

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2. Materials and methods

2.1. Protein expression and purification

We used a synthetic full-length codon optimized DNA of HDP from *Plasmodium falciparum* for recombinant protein expression (GeneScript), since rarely employed codons in *E. coli* in the target gene might result in poor gene expression in *E. coli*. Expression and purification of HDP with a 6× His-tag at the C terminus (HDP-histag) was performed as described previously [15]. Protein purity was confirmed by SDS–PAGE. For the expression of non-tag HDP (HDP-nontag), we employed the pCold expression system (Takara Bio) where protein expression is under control of the *cspA* promoter. The HDP gene fragment was ligated into the *Nde*I/*Bam*HI digested pCold IV vector. BL21(DE3) cells transformed with the pCold HDP plasmid were grown at 37 °C in 2xYT containing 100 μg/ml ampicillin. Expression was induced with 1 mM IPTG at 15 °C overnight. The recombinant protein was mainly localized in the inclusion bodies and refolded as described previously [15,21]. The refolded HDP-nontag in buffer A (50 mM CAPS-NaOH, pH 11) containing 1.5% *N*-lauroylsarcosine was applied to a HiTrap Q HP column (GE Healthcare) that was equilibrated with buffer A. The column was washed with buffer A containing 300 mM NaCl. The absorbed proteins were eluted with buffer A containing 500 mM NaCl. The fractions containing HDP-nontag were concentrated with Amicon Ultra-15 10 K filter unit (Millipore). If additional purification was required, size exclusion chromatography was used. The concentrated proteins were loaded to a Superdex 75 10/300 GL (GE healthcare) gel filtration column that equilibrated with buffer A. The purity of the fractions containing HDP-nontag was confirmed by SDS–PAGE.

2.2. Hz formation assay

Hz formation assay was performed as described with some modification [12,15]. A 10 mM fresh stock solution of hemin in *N*,*N*-dimethylformamide was prepared. The hemozoin formation assays were performed at 37 °C for 1 h in 0.5 M sodium acetate pH 5.2 containing 0.5 μM of HDP and 200 μM hemin. After incubation, the reaction mixture was centrifuged at 15,000*g* for 10 min at room temperature. The precipitations were washed with 1 mL of 0.1 M $NaHCO_3$, 2.5% SDS, pH 9.1, followed by deionized water. The final adducts were solubilized in 0.1 M NaOH and the amount of heme determined by the absorption spectra.

2.3. Spectroscopy

Electronic absorption spectra were obtained using a Shimadzu UV-3150 spectrometer. The purified HDP, which were dissolved in appropriate buffer, was incubated with hemin in *N*,*N*-dimethylformamide and then passed through a Sephadex G25 column (GE Healthcare) to remove the unbound hemin. The path-length of the cell was 0.2 or 1 cm. The HDP-ferrous heme complex was prepared by the addition of sodium dithionite in a sealed cuvette to maintain anaerobic conditions. The CO complex of HDP with ferrous heme was prepared by displacing the nitrogen-filled airspace of a sealed cuvette containing the ferrous heme–HDP complex solution with CO. FTIR spectra were measured on a JASCO FT/IR-6100. The FTIR spectra were averages of 32 scans recorded at a resolution of 2 cm^{-1}.

3. Results and discussion

In the previous study, the use of a histidine tag enabled the purification of the recombinant HDP protein (HDP-histag) from crude extracts [15]. However, the presence of the affinity tag in the recombinant protein resulted in unpredictable changes [22]. We therefore performed expression and purification from *E. coli* of the non-tagged HDP (HDP-nontag). Whole-cell lysates of *E. coli* BL21 (DE3) encoding HDP-nontag were analyzed by 15% SDS–PAGE (Fig. 1A, Lane 1). A major band appeared at approximately 23 kDa, corresponding to the expected size of the recombinant non-tagged HDP. Comparison of the soluble fraction (Fig. 1A, Lane 2) with the pellet (Fig. 1A, Lane 3) indicated that the recombinant HDP-nontag is localized mainly in inclusion bodies. The removal of the histidine tag did not change the solubility of the recombinant HDP, because HDP-histag also was found in the insoluble fraction [15,18]. The refolded proteins were purified by anion exchange chromatography (Fig. 1A, Lane 4) followed by gel filtration (Fig. 1A, Lane 5). Authenticity of the purified HDP-nontag was confirmed by N-terminal amino acid analysis and ESI-Q-TOF mass spectroscopy (Supplementary Fig. 1).

To assess the Hz formation activity of recombinant HDP-nontag, the crystalline material produced by HDP-nontag was investigated by FTIR spectroscopy. Hz can be distinguished from monomeric heme by the strong absorbance around 1200 and 1650 cm^{-1} in the FTIR spectrum; these peaks are assigned to C–O and C=O stretching vibrations, respectively, from the heme carboxylate group [23]. As shown in Fig. 1B, the crystalline material produced by HDP-nontag exhibited two strong absorbance peaks at 1210 and 1663 cm^{-1} in the FTIR spectrum, indicating the formation of reciprocal iron-oxygen bonds between two heme molecules. This result confirmed that the recombinant HDP-nontag produced crystalline heme with a structure typical of native Hz. In a Hz-production assay, HDP-nontag rapidly converted up to 38% of heme into Hz within one hour (Supplementary materials). The Hz production activity for the HDP-nontag was comparable to that previously reported for native HDP and the HDP-histag [15].

The electronic absorption spectra of HDP-nontag and HDP-histag with hemin in 50 mM MES-NaOH, pH 5.6, are shown in Fig. 1C. A broad Soret peak was found for HDP-nontag with hemin at 401 nm, while that for the HDP-histag with hemin was found at 410 nm. Thus, attaching a histidine tag to HDP influenced the electronic structure of heme in HDP. Previously, histidine-tagging was shown to interfere with heme-binding by recombinant Rv0203 and CcmE, providing an unnatural heme-binding site [19,20]. It is possible that the flexible C-terminal tag in HDP-histag interacts with hemin and also acts as an unnatural heme-binding site. In the case of the His-tagged Rv2023, the histidine-tag bound to the open face of the heme molecule and changed the electronic properties of the heme group [19]. Although the coordination structure of heme in HDP is not clear, the C-terminal histidine tag may interact with heme in this protein. Therefore, we employed HDP-nontag for further characterization.

HDP is thought to be involved in the formation of a heme dimer, serving as a seed for crystal growth of Hz via the multiple heme-binding sites in HDP. Isothermal calorimetric analysis of the recombinant HDP-histag detected the presence of 2.7 heme-binding sites per HDP-histag molecule [15]. To determine the stoichiometry of the heme-binding in HDP-nontag, we monitored absorbance changes at 414 nm (Fig. 2). Because HDP can produce Hz at pH 5.2 or lower, titrations with hemin were carried out at pH 7.0. The hemin titration assay clearly indicates that hemin bound to HDP-nontag with a stoichiometry of 2:1 (Fig. 2, inset). Therefore, HDP-nontag contains at least two independent heme-binding sites. The discrepancy in heme-binding capability between HDP-nontag and HDP-histag presumably reflects interaction of the histidine tag with heme. The Hz production activity for the recombinant HDP-histag is more consistent with that of native HDP [15]. However, introduction of the histidine tag apparently causes unpredictable changes in the heme coordination structure of HDP.

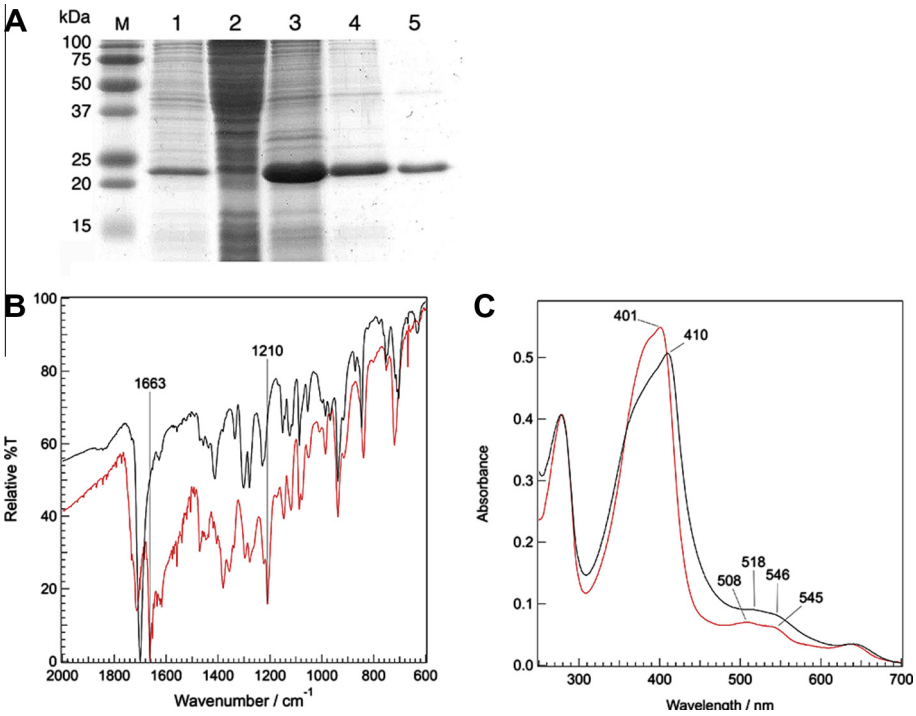

Fig. 1. Purification and characterization of HDP-nontag. (A) SDS–PAGE analysis for HDP-nontag purification. Lane 1, total cell protein from HDP-nontag-encoding *E. coli* BL21(DE3) after IPTG induction; Lane 2, soluble protein from the supernatant; Lane 3, insoluble protein from the pellet of centrifuged cells; Lane 4, eluate from HiTrap Q HP anion exchange column; Lane 5, eluate from Superdex 75 gel filtration column. M, molecular weight marker. (B) FTIR spectra of the recombinant HDP-nontag-produced crystalline material (red line) and hemin (black line). (C) Electronic absorption spectra of hemin-treated HDP-nontag (red line) and HDP-histag (black line). The sample concentration was 50 μM in 100 mM MES-NaOH, pH 5.6, at room temperature. The path-length of the cell was 0.2 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

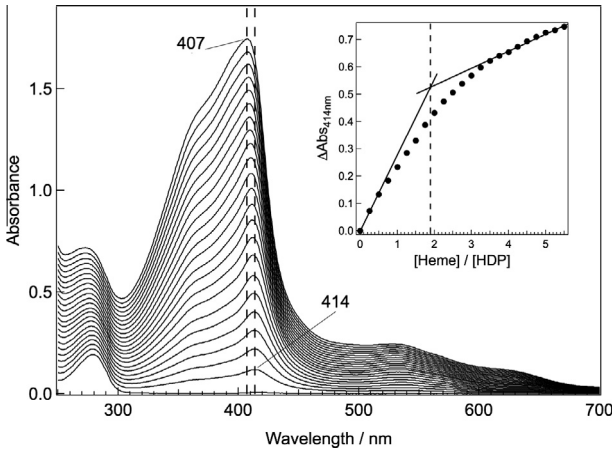

Fig. 2. Electronic absorption spectra of HDP-nontag after the addition of hemin at amounts of up to 5.5 mol equivalents of protein. The inset shows titration curves of HDP-nontag after the addition of increasing amounts of hemin, as monitored by the peak absorbance at 414 nm. The sample concentration was 5 μM in 200 mM MOPS-NaOH, pH 7.0, at room temperature. The path-length of the cell was 1 cm.

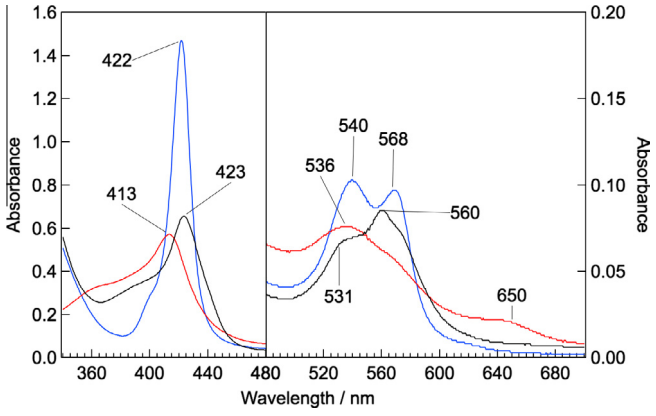

Fig. 3. Electronic absorption spectra of ferric (red line), ferrous (black line) and ferrous-CO (blue line) forms of heme bound to HDP-nontag. The sample concentration was 10 μM in 50 mM MOPS-NaOH, pH 7.0, at room temperature. The path-length of the cell was 0.2 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Since the electronic absorption spectra for the reduced form of heme protein provide insight into the heme coordination structure, we prepared HDP-nontag with two molar equivalents of heme under reduced conditions. As shown in Fig. 3, the reduction of heme iron shifted the Soret absorbance from 413 to 423 nm and slightly increased the heme iron extinction relative to the oxidized form. The reduced form of HDP-nontag with heme exhibited a visible peak at 560 nm with a shoulder at 531 nm, indicating that multiple spin and/or coordination states were present [24]. When the purified, reduced complex of HDP-nontag with heme was exposed to CO, the Soret peak shifted from 423 to 422 nm, and the Soret band increased in intensity. The spectroscopic features of HDP-nontag with the reduced heme under CO atmosphere were similar to those of CO adducts of hemoglobin, myoglobin, and a variety of other histidine-ligated heme proteins [25]. In those molecules, cysteine-thiol ligation to the heme iron gives rise to a distinctive absorption band at approximately 450 nm for the CO adducts [26]. The results of the absorption spectra suggest that the heme-binding site of HDP-nontag consists of at least one histidine residue. The amino acid sequence of HDP contains nine histidine residues, of which seven are conserved among different *Plasmodium* species [15].

Determination of the role of histidine residue(s) in Hz formation will require further investigation.

In this study, we characterized recombinant HDP-nontag purified from *E. coli*. The pCold system provided increased expression of HDP, and the protein was localized mainly in the inclusion bodies. Production without the histidine tag did not affect the solubility of the recombinant HDP. However, comparison of the electronic absorption spectrum between the HDP-nontag and HDP-histag revealed that the C-terminal histidine tag influenced the electronic structure of the bound heme. Expression of HDP without the histidine tag did not alter Hz formation [15,18], and analysis of the intact, non-tagged recombinant protein will be important for understanding the heme coordination structure of native HDP. A heme titration assay demonstrated that HDP-nontag binds two molecules of heme per protein molecule. Purified HDP-nontag gave precise information about the heme-binding site for the formation of Hz. A possible reaction mechanism is as follows: the two hemes bind to histidine residue(s) in HDP; reciprocal iron-oxygen bonds are formed between the two heme molecules; and then the heme dimer is released from the protein. The electronic absorption spectrum of HDP-nontag with ferrous–CO heme indicates that the heme-binding to a histidine residue in HDP plays an important role in the formation of the heme dimer that acts as a seed crystal for Hz. The detailed mechanisms of HDP-mediated Hz formation will be the subject of further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.100.

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