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Efficient refolding and functional characterization of *Pf*AMA1(DI+DII) expressed in *E. coli*

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ABSTRACT

Apical membrane antigen 1 (AMA1) is a surface protein of *Plasmodium sp.* that plays a crucial role in forming moving junction (MJ) during the invasion of human red blood cells. The obligatory presence of AMA1 in the parasite lifecycle designates this protein as a potential vaccine candidate and an essential target for the development of novel peptide or protein therapeutics. However, due to multiple cysteine residues in the protein sequence, attaining the native fold with correct disulfide linkages during the refolding process after expression in bacteria has remained challenging for years. Although several approaches to obtain the refolded protein from bacterial expression have been reported previously, achieving high yield during refolding and proper functional validation of the expressed protein was lacking. We report here an improved method of refolding to obtain higher quantity of refolded protein. We have also validated the refolded protein's functional activity by evaluating the expressed AMA1 protein binding with a known inhibitory peptide, rhoptry neck protein 2 (RON2), using surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC).

1. Introduction

Malaria is one of the widespread infectious diseases that cause risk to nearly half of the global population and responsible for the death of almost half a million people every year [1]. The parasite responsible for this disease is *Plasmodium sp.,* the deadliest of which is *Plasmodium falciparum* [1]. In 2018, 94% of global deaths due to malaria were reported from sub-Saharan Africa, the most malaria-endemic region where 99.7% of malaria infection was due to *P. falciparum.* The parasite's widespread resistance towards the frontline antimalarial therapy accounts for the delayed control over malaria [2]. The best treatment available until now for *P. falciparum* is the artemisinin combination therapy (ACT). But in some regions of south-east Asia, several of the *P. falciparum* strains have already started showing resistance against ACTs [1,3]. Therefore, there is a continual and urgent need to develop alternative yet effective antimalarial therapeutics to eradicate malaria.

This protozoan parasite possesses a myriad of proteins, the majority of which are localized either on the surface or in the parasites' secretory organelles. Several of these proteins are essential for red blood cell invasion [4]. The invasion mechanism involves the formation of a moving junction (MJ) between the surface of the parasite and the host cell membrane [5]. The MJ is found to be conserved in all apicomplexan parasites. Two parasite proteins, apical membrane antigen 1 (AMA1) and rhoptry neck protein 2 (RON2), are responsible for the MJ formation during the host cell invasion [6]. Therefore, AMA1-RON2 protein-protein interaction is a potential target to stop the parasite invasion process. The AMA1-RON2 protein-protein interaction interface as an antimalarial therapeutic target had been further validated by a peptide inhibitor R1, derived from peptide phage display library, and the soluble RON2 ectodomain (RON2ed), which successfully inhibited the red blood cell invasion by disrupting AMA1-RON2 interactions [7–9]. Reported crystal structures revealed that both R1 and RON2ed peptides bound to the same hot-spot of the AMA1 protein for the inhibition [10]. Therefore, when designing a new peptide or protein inhibitor against AMA1, it is crucial to keep in mind that the inhibitor should target the correctly folded and functional AMA1 hot-spot.

The AMA1 protein is also an essential protein for the parasite's survival, as dispensing off the AMA1 gene using 'knock-out' plasmids thwarted normal parasite growth [9]. Moreover, AMA1 anti-sera successfully inhibited the parasite's erythrocytic invasion, indicating AMA1

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to be a potential vaccine candidate [4]. Immunization studies revealed that reduced and alkylated AMA1 failed to provide a protective immune response, suggesting that the formation of correct disulfide bonds is obligatory to maintain the structural integrity of the AMA1 functional epitopes [11].

A correctly folded AMA1 can be obtained from insect cell or yeast cell expression [6,12,13]. However, to eliminate the aberrant glycosylation in the protein expressed from eukaryotic cells, the AMA1 protein was expressed in bacteria by several groups [4,14–16]. But the difficulty with the *E. coli* expression system was to obtain the correctly folded and functionally active AMA1 protein in reasonably good quantity, which indeed is the bottleneck for any refolding process. Herein, we report the successful refolding of the AMA1 protein expressed in bacteria and validation of the refolded protein's functional activity by evaluating its binding with the chemically synthesized extracellular peptidic domain of RON2 by SPR and ITC.

2. Materials and methods

2.1. Overexpression of 3D7 PfAMA1 (DI + DII) in E. coli

Gene synthesis and sub-cloning were done to obtain the expression plasmid coding for amino acids 104–438 of 3D7 *Pf*AMA1 spanning the first two (DI + DII) domains. The plasmid was purchased from GenScript (NI, USA) that carried the pET 28a (+) backbone (Novagen, Merck). The gene of interest (GOI) also had N-terminal His_6-tag to facilitate purification using Ni-affinity chromatography. Protein expression was carried out in *Escherichia coli* BL21 (DE3) RIL cells. Briefly, the cells carrying the expression plasmid were grown in LB broth until the OD_{600} reached 0.6, and then the protein expression was induced with 1 mM IPTG for 4 h at 37 °C. The induced cells were pelleted by centrifugation (at 5000 g) and stored at −80 °C till further use.

2.2. Solubilization and affinity purification

The expressed protein was insoluble and formed inclusion bodies. To extract the expressed protein from inclusion bodies, cell pellets were re-suspended in 100 ml ice-cold buffer A [6 M guanidine hydrochloride (Gu.HCl), 20 mM Tris, pH 8.0, 250 mM NaCl, 2 mM β-mercaptoethanol (BME) and lysozyme (0.25 mg/ml)]. The cell suspension was stirred overnight at 4 °C and then sonicated with 10 short bursts of 5s using the lowest power setting to homogenize the cells completely. The cell lysate was centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was applied onto a 5 ml Ni-NTA agarose column (Qiagen) equilibrated with buffer A. After allowing the His-tagged protein to bind for approximately 1 h, the column effluent was discarded, and the column was washed with 5 column volumes of buffer B (8 M urea, 20 mM Tris pH 8.0, 250 mM NaCl, 2 mM BME and 20 mM imidazole). The bound protein was eluted with 5 column volumes of buffer C (8 M urea, 20 mM Tris pH 8.0, 250 mM NaCl, 2 mM BME and 250 mM imidazole).

2.3. Purification of the reduced polypeptide by HPLC

The eluent containing the partially reduced *Pf*AMA1 (DI + DII) polypeptide obtained from Ni affinity column was entirely reduced by treatment with 40 mM Tris (2-carboxyethyl)phosphine (TCEP) for 30 min at pH 7.46, acidified and purified by reverse-phase HPLC. The reduced polypeptide was then eluted through a C4 Waters reverse-phase column (5 μm, 10 mm × 250 mm) using a linear gradient 20–40% of buffer B′ in buffer A' (buffer A' = 0.1% TFA in water; buffer B' = 0.08% TFA in acetonitrile) over 40 min with a flow rate of 5 ml/min. The pooled fractions, containing the pure *Pf*AMA1 (DI + DII) polypeptide, were lyophilized and stored at 4 °C until further use.

2.4. Refolding of the lyophilized polypeptide

The lyophilized polypeptide was dissolved in buffer D (6 M Gu.HCl, 20 mM Tris, pH 8.0, 100 mM NaCl and 10 mM DTT) to a final concentration of approximately 1 mg/ml. The reduced polypeptide was then dialyzed against 50 times higher volume of buffer E [6 M urea, 20 mM Tris, pH 8.0, 100 mM NaCl, 2 mM reduced glutathione (GSH), and 0.5 mM oxidized glutathione (GSSG)] for 2 h at 4 °C. Next, a step-wise dialysis against gradually decreasing concentration of urea (4 M, 2 M, 0.5 M) was performed over a period of 24 h. Apart from urea, the concentrations of all other buffer components were left unaltered. The dialysis was continued in 0.5 M urea containing buffer for approximately 12 h, followed by buffer containing no urea for 24 h at 4 °C. The formation of five disulfide bonds in the refolded protein was monitored by LCMS. In order to remove the redox reagents the refolded protein was further dialyzed against 20 mM Tris and 100 mM NaCl, while gradually decreasing the pH to 7.8. The insoluble misfolded protein aggregates obtained during the refolding process were removed by centrifugation. The refolded protein was then concentrated and loaded onto a HiLoad 16/600 Superdex 200 pg column (Cytiva –GE healthcare Life Sciences, USA) equilibrated with buffer F (20 mM Tris, pH 7.8 and 100 mM NaCl) to remove the soluble aggregates if any. The eluted pure fractions were then analyzed and pooled.

2.5. Chemical synthesis of PfRON2ed

39-residue *Pf*RON2ed peptide (Asp2021-Ser2059) was synthesized by step-wise Fmoc chemistry solid phase peptide synthesis (SPPS) in an automated peptide synthesizer (Tribute UV-IR, Protein Technologies Inc. USA). The peptide was synthesized on 2-chlorotrityl chloride (CTC) resin. Disulfide bond formation of the peptide was achieved by air oxidation in Tris buffer at pH 8 [17]. The folded peptide was purified by reverse-phase HPLC and lyophilized. The lyophilized peptide was reconstituted in buffer F (20 mM Tris pH 7.8 and 100 mM NaCl) before performing binding studies using ITC experiments and in buffer G (10 mM Phosphate buffer saline, pH 7.4 with 0.005% Tween-20 and 40 μM EDTA) for SPR experiment.

2.6. Growth inhibition activity assay of chemically synthesized PfRON2ed

Plasmodium falciparum 3D7 cell line culture was synchronized tightly before growth inhibition assay. Intraerythrocytic late trophozoite or early schizont stage with parasitemia 0.3% were subjected to treatment with varying concentrations (1 nM, 10 nM, 100 nM, 1000 nM, 10,000 nM and 50,000 nM) of chemically synthesized *Pf*RON2ed in nutrient-rich complete media and allowed to incubate for 72 h as described previously [17,18]. Upon completing the incubation period, the standard Giemsa counting assay was performed by preparing a thin smear by taking a 4 μL palette culture after centrifugation. The thin smears were prepared on slides for each treated concentration, followed by fixation with methanol. Staining with Giemsa solution was done and slides were observed under a microscope using 100× oil objective. From random adjacent microscopic fields, 2000 red blood cells (RBCs), including infected RBCs (iRBCs), were counted for each concentration and the final percentage parasitemia was calculated. This assay was performed twice for reproducibility.

2.7. AMA1-RON2ed binding studies by SPR

SPR measurements were carried out using a BI-4500AP SPR Instrument. The refolded His-tagged AMA1 protein (3.85 μM, pH 7.80) was immobilized over a Nickel-NTA chip leaving one flow cell as the reference channel. Binding assays of AMA1-RON2 was performed at 25 °C using buffer G (10 mM Phosphate buffer saline, pH 7.4 with 0.005% Tween-20 and 40 μM EDTA) as the running buffer. To obtain the sensorgrams a series of different concentrations of RON2 peptide dissolved

in running buffer were injected at a constant flow rate of 30 μl/min. For the peptide dissociation, sample injections were stopped and the running buffer was flowed at the same flow rate. To obtain the final sensorgrams, the sensorgram of the control flow cell was subtracted from sensorgrams of the ligand flow cells. The interactions were analyzed using the BI-data analysis software by fitting the data to a 1:1 Langmuir adsorption binding isotherm. Three repeats of the AMA1-RON2 binding experiments were performed with the same batch of refolded AMA1 protein and folded RON2 peptide. The average K_D value from the three repeats obtained was 21.88 ± 1.89 nM. The kinetic data for every repeat experiment are shown in Supporting Information, Figure S1-S3 and Table S2.

2.8. AMA1-RON2ed binding studies by ITC

ITC measurements were performed using a MicroCal iTC200 instrument. The protein concentration was 10 μM, while the ligand (*Pf*RON2ed) concentration was 200 μM (concentration of the peptide ligand was calculated with respect to its dry weight). Initially, 200 μl of the AMA1 protein was loaded in the sample cell, and the ligand was then titrated into the sample cell (17 injections of 2 μl each in 150 s intervals at 25 °C). The heat of dilution of the ligand in buffer was subtracted from the raw data, and a single-site binding model was used to fit the ITC data. The data fitting was performed using Origin software (Microcal). The binding study was conducted in three repeats (including one repeat with a different batch of refolded AMA1) with freshly refolded AMA1 to check the reproducibility of the binding of the RON2 peptide with refolded AMA1. The average K_D value from the three repeats obtained was 121.9 ± 20.1 nM. The thermodynamic data for every repeat experiment are shown in Supporting Information, Figure S4-S6 and Table S1.

3. Results

3.1. Expression, purification and refolding of PfAMA1(DI + DII)

*Pf*AMA1 protein consists of three domains. The first two domains (DI and DII) participate in binding to its receptor rhoptry neck protein 2 (RON2). We expressed the *Pf*AMA1 (DI + DII) in *E. coli* with an N-terminal His_6-tag (Fig. 1). After expression, total protein was extracted from the inclusion bodies under denaturing conditions and purified by Ni-affinity chromatography (Fig. 2A) to obtain the desired His-tagged *Pf*AMA1 (DI + DII) polypeptide.

The His-tagged *Pf*AMA1 (DI + DII) polypeptide obtained from Ni-affinity chromatography contained partially reduced polypeptide forming cross-disulfide adducts with beta-mercaptoethanol (BME) over time. Hence, the *Pf*AMA1 (DI + DII) polypeptide was reduced entirely using 40 mM TCEP and further purified by reverse-phase HPLC before refolding (Fig. 2B).

We refolded the purified and lyophilized polypeptide by step-wise dilution at pH 8 at 4 °C in the presence of reduced and oxidized glutathione, as described in the *Materials and Methods* section. After each step of the refolding process, protein samples were loaded onto the gel (Fig. 3A). The formation of five disulfide bonds during the refolding process was confirmed by LCMS, as shown in Fig. 3B. The optimized refolding protocol was reproducible within the pH range of 7.8–8.4.

During the refolding process, most of the misfolded proteins precipitated in the dialysis bag, while some aggregates that remained soluble were removed by size-exclusion chromatography (SEC). We started the refolding process with 25 mg of purified reduced AMA1 polypeptide. The protein yield was approximately 9 mg and 4.5 mg after refolding and final SEC, respectively. In order to test the reproducibility of the protocol we performed the refolding experiment more than three times with different batches of samples that produced consistent results.

3.2. Chemical synthesis, oxidative folding and biological activity of RON2ed

To validate the refolded *Pf*AMA1 protein's functional activity, we needed to synthesize the peptide ligand (RON2ed) that is known to bind with *Pf*AMA1 protein. We chemically synthesized the 39 mer RON2ed polypeptide by Fmoc-chemistry SPPS using an automated peptide synthesizer [17]. The chemically synthesized peptide was then allowed to undergo oxidative folding under air oxidation conditions in buffer H (2 M Gu.HCl and 100 mM Tris, pH 8.4) for 18 h. After the folding with a disulfide bond formation was complete, as monitored by LCMS (Fig. 4A),

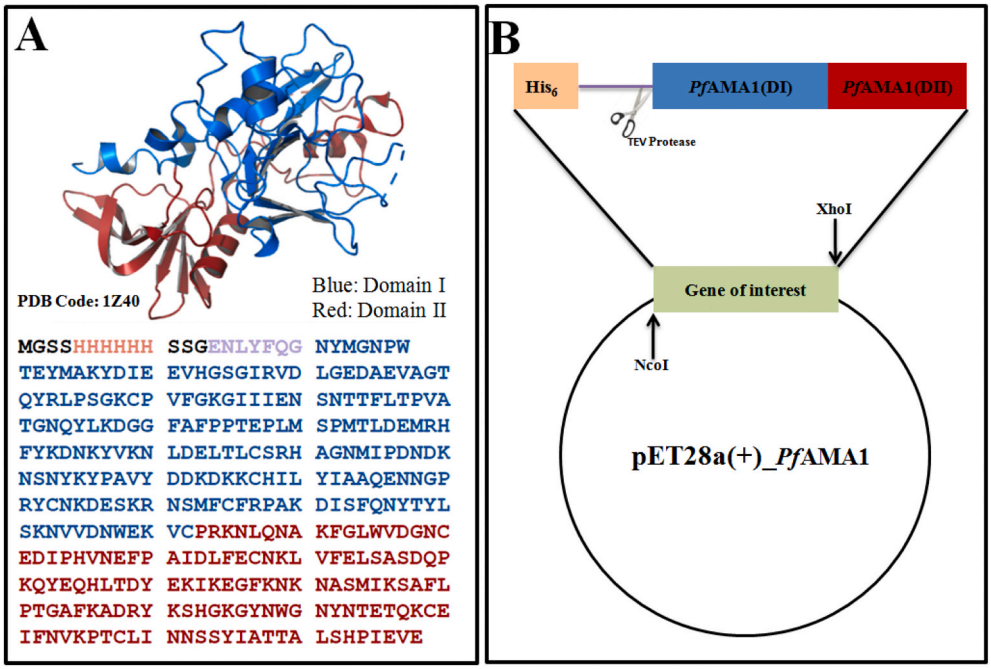

Fig. 1. (A) The sequence and the tertiary structure of the *Pf*AMA1 (DI + DII) protein; (B) Plasmid construct containing 6xHis tag and TEV cleavage site for the *Pf*AMA1 (DI + DII) expression. The 6xHis tag was not removed from the fusion protein in our study.

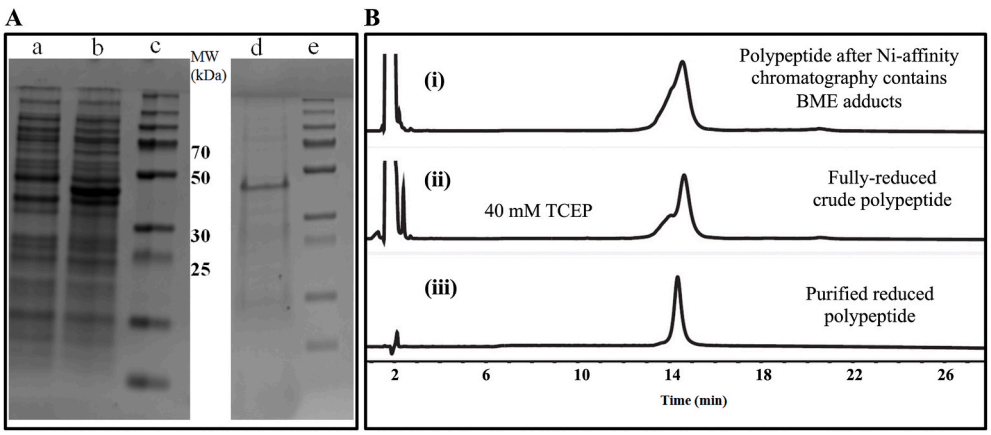

Fig. 2. (A) Gel image showing protein expression (left) and purified denatured *Pf*AMA1 (DI + DII) polypeptide from Ni-affinity chromatography (right). Lane a: uninduced, lane b: induced, lane c and e: molecular weight markers, lane d: eluent from Ni-affinity column; (B) LC chromatogram of (i) eluent from Ni-affinity column, (ii) eluent after complete reduction of disulfides with 40 mM TCEP, (iii) fully reduced polypeptide after purification by reverse-phase HPLC. The observed mass of the purified polypeptide was 40450.36 ± 0.33 Da (mass calculated: 40449.28 Da).

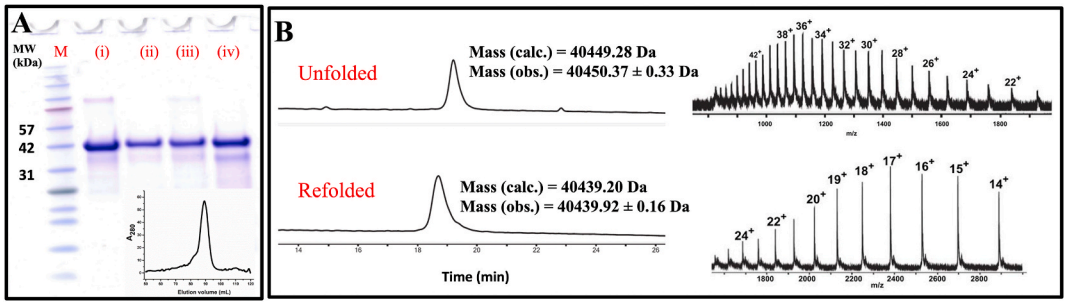

Fig. 3. (A) Gel image of the protein after each step of refolding process M: marker lane, (i) lyophilized unfolded protein, (ii) refolded protein, (iii) dialyzed refolded protein in ITC buffer F, (iv) refolded protein obtained after Size exclusion Chromatography [inset: Gel filtration profile in S200 column]; (B) LCMS of the unfolded and fully-reduced *Pf*AMA1 (DI + DII) [Calculated mass (average isotope) = 40449.28 Da, Observed mass (average isotope) = 40450.37 ± 0.33 Da] and refolded *Pf*AMA1 (DI + DII) [Calculated mass (average isotope) = 40439.20 Da, Observed mass (average isotope) = 40439.92 ± 0.16 Da].

we purified the folded peptide using reverse-phase HPLC and lyophilized. The *in vitro* growth inhibition activity (GIA) assay confirmed that the chemically synthesized folded *Pf*RON2ed was fully functional, as it inhibited the *P. falciparum* (3D7) parasite growth successfully (Fig. 4B).

3.3. Validation of functional activity of the expressed PfAMA1(DI + DII)

We demonstrated the refolded AMA1 protein's functional activity using ITC and SPR, wherein the binding of *Pf*AMA1 with its ligand, *Pf*RON2ed, was studied. We previously showed by growth inhibition activity assay that the chemically synthesized *Pf*RON2ed was biologically active. The peptide successfully inhibited the merozoite invasion into the red blood cells. Here we showed that the same *Pf*RON2ed peptide bound to *Pf*AMA1 (DI + DII) at best affinity of 20.9 nM (in SPR) and 93.5 nM (in ITC) validating the fact that the expressed *Pf*AMA1 (DI + DII) protein obtained using modified refolding conditions described here was indeed functionally active (Fig. 4C and D and Table S1). The yield and the binding activity of the refolded protein obtained by this method were compared with the AMA1 obtained from other expression systems as shown in Table 1 and Table S2, respectively.

4. Discussions

The MJ formation between the apical end of *Plasmodium falciparum* parasites and the host cell surface is typical in all *Apicomplexan* parasites [13]. AMA1 and RON2 are the two essential proteins involved in the formation of the MJ. Therefore, the disruption of these two proteins' interactions by designed peptide inhibitors could stop the merozoite invasion into the red blood cells [15]. To validate any peptide inhibitor's binding, we require a functionally active AMA1 protein with which the protein-ligand complex will be formed. Moreover, immunization studies have shown that the recombinant AMA1 protein is a potential vaccine candidate [19]. However, to generate a useful neutralizing antibody by vaccination, the recombinant AMA1 protein must have the correct disulfide linkages, similar to the native protein, as, during the immunogenicity study, the antibody elicited by reduced and alkylated AMA1 protein was unable to stop the parasite invasion [20]. Therefore, generating a properly folded AMA1 protein, having correct disulfide bond combinations, is an important first step for vaccine or inhibitor design.

The receptor-binding domains of *Pf*AMA1, i.e., *Pf*AMA1 (DI + DII), has a total of ten cysteine residues that form five disulfide bonds in its folded form. Therefore, the most challenging step in the refolding process is to create the correct disulfide combinations. In-vitro refolding often results in kinetically trapped misfolded protein aggregates. When the refolding of the AMA1 protein was attempted following previously reported protocols, no binding or very low binding was observed with the inhibitory peptide (*Pf*RON2ed) (data not shown). This lack of binding could be attributed to the presence of more misfolded than the rightly folded proteins. Therefore, in most reported cases, the AMA1 protein was derived from insect cells or yeast cells, resulting in in-situ correctly disulfide-bonded protein to avoid such an issue. However, the chances of getting aberrant glycosylation in the folded proteins that may interfere with the downstream applications limit the use of insect cells or yeast cells as the expression system [21,22]. In order to rectify this problem, Gupta et al. [4] first expressed the AMA1 protein in bacterial expression system followed by refolding. Even though the refolded protein obtained by this method successfully provided an active immunogen, the protein's functional activity remained elusive to a major extent. Moreover, the concentration at which the protein refolding was carried out in the reported protocols was extremely low, necessitating large volumes of refolding buffers for dialysis.

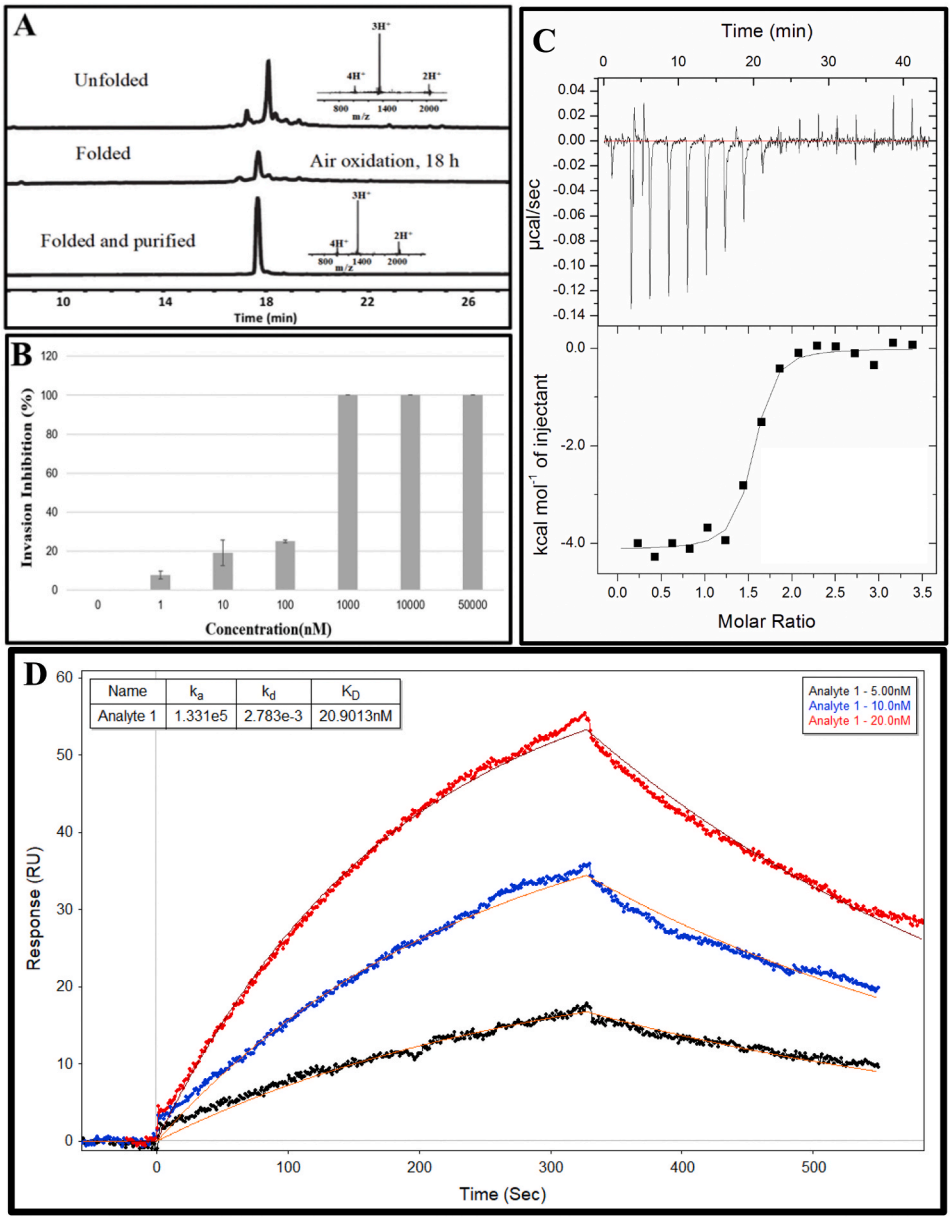

Fig. 4. (A) Folding of 39 mer *Pf*RON2ed peptide; (top) LCMS chromatogram of the chemically synthesized unfolded *Pf*RON2ed (crude) (Calculated mass (most abundant isotopologue) 4063.01 Da, Observed mass (most abundant isotopologue) = 4063.02 ± 0.02 Da); (middle) folded *Pf*RON2ed (crude); (bottom) purified folded *Pf*RON2ed (Calculated mass (most abundant isotopologue) = 4060.99 Da, Observed mass (most abundant isotopologue) = 4061.02 ± 0.01). (B) In vitro parasite growth inhibition activity (GIA) assay of chemically synthesized *Pf*RON2ed (C) Binding of *Pf*AMA1 (DI + DII) (Batch 1) vs. *Pf*RON2ed in ITC experiment. (Top panel) the raw data; (Bottom panel) the binding isotherms created by plotting the integrated heat against the molar ratio of the peptide. (D) A representative SPR sensorgrams with best analytical fit of the binding of *Pf*AMA1 (DI + DII) with *Pf*RON2ed.

Table 1
Comparison of the yield of the refolded AMA1 protein obtained in this work with other reported protein expressions.

Protein [references]	Expression system	Yield per liter of culture
*Pf*AMA1 (refolded) (This work)	Bacterial (*E. coli*)	4.5 mg
*Pf*AMA1 (refolded) [20]	Bacterial (*E. coli*)	0.75–1 mg
*Pf*AMA1 (refolded) [4,14–16,19]	Bacterial (*E. coli*)	Not mentioned
Plasmodium vivax AMA1 (refolded) [23]	Bacterial (*E. coli*)	Not mentioned
*Pf*AMA1 (folded) [10]	Insect (Sf9 cells)	3 mg
*Pf*AMA1 (folded) [10]	Yeast (*P. pastoris*))	20 mg

Furthermore, the lack of clarity on the refolding efficiency or the functional assay of the refolded protein was evident from other reports as well [14–16]. Therefore, to obtain the rightly folded AMA1 protein from a bacterial expression system that can be used for binding assay with its inhibitor (for example, the extracellular peptidic domain of *Pf*RON2), we had to take a different approach.

Our primary focus was to obtain the correctly folded *Pf*AMA1 (DI + DII) protein with a satisfactory yield and eliminate the misfolded protein aggregates, which can interfere with the protein activity. Therefore, unlike most of the previously reported protocols that involved rapid dilution, we chose a step-wise dialysis technique under redox condition for the refolding with concomitant formation of five disulfide bonds of the protein with a starting concentration of 1 mg/ml while refolding. The insoluble misfolded protein aggregates formed during the refolding process were removed by centrifugation, and the soluble misfolded proteins were removed by purification via size-exclusion chromatography. The refolded *Pf*AMA1 protein showed a 10 Da mass decrease from the unfolded reduced protein in LCMS, indicating formation of five disulfide bonds. The ESI-MS data of the refolded protein showed reduced charge state distribution compared to its unfolded form – the typical characteristic pattern usually observed for a globular folded protein (Fig. 3B). The purified protein yields were highly consistent among multiple batches of refolding experiments. The yield of the folded and purified PfAMA1 protein obtained using this approach was nearly five times better than previously reported results from bacterial expression

systems (Table 1, entry 2) and was comparable to the same protein obtained from Insect cells expression systems (Table 1, entry 5). The *Pf*AMA1 protein obtained from the highly reproducible refolding protocol described above was proven to be fully active, as evident from the binding of the refolded protein with the inhibitory peptide (*Pf*RON2ed) determined by isothermal titration calorimetry and surface plasmon resonance (Supporting Information, Figure S1-S6 and Table S1). The batch wise reproducibility of binding event and 1:1 binding mode of the AMA1 and RON2ed ligand was evident from the ITC experiment with an observed K_D value ranging from 93.5 to 136.8 nM (Table S1). The considerable variation in the observed K_D values in the ITC experiments arises due to the experimental uncertainties in the determination of the dissociation constant, which is the limitation of the ITC technique. The average K_D value obtained from three repeats in SPR experiment (21.88 $\pm$ 1.89 nM) correlated well with the reported K_D value observed from the binding study using AMA1 obtained from insect cell expression system (entry 3, Table S2).

In conclusion, we have adopted a modified and reproducible approach for the high-yield expression of *Pf*AMA1 (DI + DII) from *E. coli,* using a hassle-free refolding procedure. The protein obtained by this approach is the functionally active AMA1 protein, which can be used as a vaccine candidate or can be targeted for the development of novel inhibitors for the disruption of the AMA1-RON2 interactions to inhibit the parasite invasion into the red blood cells.

Author Statement

AB, SRK, MKB and KM designed experiments. All authors except MKB and KM performed experiments. All authors analyzed the data. AB, SRK, MKB and KM wrote the manuscript. All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100950.

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