Erythrocytic stage dependent regulation of oligomerization of *Plasmodium* ribosomal protein P2

**Sudipta Das, Sudarsan Rajagopal, Subramanian Sivakami and Shobhona Sharma***

1Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India, 2Department of Life Sciences, University of Mumbai, Mumbai, India

*Corresponding author: Shobhona Sharma (sharma@tifr.res.in)

Running title: Developmentally regulated oligomerization of *Plasmodium* P2 protein.

**Keywords:** *Plasmodium falciparum*, acidic ribosomal protein P2, regulation of oligomerization, SDS-resistance, DTT-resistance, rhodamine-labeled P2.

**Background:** *Plasmodium falciparum* P2 (PfP2) protein plays non-ribosomal roles through SDS and DTT-resistant oligomerization.

**Result:** For SDS and DTT-sensitive oligomerization, 53rd cysteine of PfP2 plays important role.

**Conclusion:** DTT and SDS resistant oligomerization of PfP2 was propagated by differentially expressed parasite proteins.

**Significance:** Analysis of regulation of PfP2 oligomerization in parasite infected erythrocyte may help in understanding the export of P2 to erythrocyte surface.

**ABSTRACT**

The eukaryotic 60S-ribosomal stalk is composed of three P ribosomal proteins (P0, P1 and P2) which associate in a pentameric structure [2P1, 2P2, P0] in the ribosome. The *Plasmodium falciparum* protein P2 (PfP2) appears to play non-ribosomal roles. It gets exported to the infected erythrocyte (IE) surface at 30 hrs post merozoite invasion (PMI), concomitant with extensive oligomerization. Here we present certain biophysical properties of PfP2. Recombinant P2 (rPfP2) protein showed SDS-resistant oligomerization, which could be significantly abolished under reducing conditions. However, the protein continued to oligomerize even when both the cysteine residues were mutated, and with up to 40 amino-acids (aa) deleted from the C terminal end. Gel filtration profiles of rPfP2 and mutant proteins showed anomalous migration and this anomaly was removed through the C-terminal 40 aa but not 20 aa deletion. CD analysis of rPfP2 showed largely α-helical and random coil domains. The SDS and DTT resistant oligomerization was studied further as it occurred in a development specific manner in *Plasmodium*. In a synchronized erythrocytic culture of *P. falciparum*, the PfP2 protein was detected as part of the ribosomal complex (~96 kDa) at 18 and 30 hrs PMI, and was SDS-sensitive. However, at 30 hrs, large amount of SDS-sensitive aggregates of > 600 kDa were also seen. At 30 hrs PMI, each of the parasite, IE-cytosol and IE-ghost contained 60-80 kDa PfP2 complexes, which resolved to a single 65 kDa species on SDS-PAGE. Tetramethyl-rhodamine labeled rPfP2 protein exhibited DTT and SDS resistant oligomerization when treated with *P. falciparum* parasite extracts only from 24-36 hrs PMI, and multiple proteins seem to be required for this oligomerization. Understanding the developmental regulation of the oligomerization of PfP2 protein may help in the elucidation of the novel structure-function relationship in the export of PfP2 to the red cell surface.

**INTRODUCTION**

The ribosomal stalk consists of certain proteins, functionally conserved in all organisms (1-3). In eukaryotes, the stalk is composed of three types of P-proteins, P0, P1 and P2. The P0 protein, equivalent to the L10 protein in prokaryotes, forms the base of the stalk and directly interacts with the 28S rRNA, and constitutes the binding site for the two protein dimers, (P1- P2)2 (4-6). In eukaryotes the dimers are made of two independent polypeptides, P1 and P2, which is different from that in prokaryotes, which contain two to three homodimers of L12 protein (4, 6). In lower eukaryotes, such as yeast and *Trypanosoma*, two additional subgroups are distinguished, comprising...
the P1α, P1β, P2α, and P2β proteins (7, 8) while an additional P3 protein has been recognized in plants (9).

It is believed that P protein-pentameric complex binds to eukaryotic 28S rRNA and plays an important role in the GTPase-associated center of eukaryotic ribosomes (5, 10-11). When added to prokaryotic 23S rRNA, this P-protein complex changes the specificity of the ribosome to the eukaryotic elongation factors (12). This strong dependence on the P0-P1-P2 complex for the factors accessibility suggests a direct interaction between the protein complex and elongation factors. It has also been suggested that the pentameric P-complex modulates the structures of the sarcin/ricin domain of 23S/28S rRNA and makes them accessible to eukaryotic elongation factors (13). It has been reported that binding of P1 and P2 to P0 protein induces the binding activity of P0 to rRNA (10). Through several deletion constructs, it was shown that the C-terminal half of P0 protein contains two neighboring sites for P1-P2 heterodimers (14). In yeast, each of the P1 or P2-null mutants is viable in rich medium and no significant effects are seen in the rates of peptide bond formation, although the protein synthesis and growth rates are reduced (15). Heterogeneity of P-proteins in ribosomal composition has been observed, and ribosomes from stationary phase deficient in P1/P2 proteins have been reported (15). It is of interest that the pattern of protein expression in the absence of P1 and P2 proteins is distinct from that in the presence of these acidic proteins (16). It was shown that such a differential expression pattern was not due to translation error or termination suppression, but was postulated to be due to differential translation modulation, and/or due to extra-ribosomal properties of these acidic proteins (16).

Ribosomal proteins are known to play varied roles besides protein synthesis (17). We have earlier demonstrated that P0 protein plays a protective role at the merozoite surface (18-20). In Neisseria gonorrhoeae, the functional orthologue of P2 (L12), is shown to be surface exposed, and has been implicated in cell invasion (21). There have been associations of ribosomal protein expression with cancer, but those have been subscribed to altered cellular protein synthesis (22). In Plasmodium, neither P1 nor P2 protein is likely to be vital for the ribosomal functions, because in a complementation study in Saccharomyces cerevisiae, we have demonstrated that ribosomes containing just PfP0 (without any P1/P2 proteins) were capable of synthesizing proteins (23). We have not been able to knock-out Plasmodium P2 protein (Tewari and Sharma, unpublished results), and therefore surmise that P2 protein may possess important extra-ribosomal functions.

A non-ribosomal role of Plasmodium P2 protein certainly appears to exist, since a translocation of P2 protein, but not P0 or P1 proteins, to the infected erythrocyte (IE) surface during early cell division has been observed recently (24). Moreover, an unusual cell-cycle arrest of Plasmodium occurs when IEs are treated with a panel of anti-PfP2-specific monoclonal antibodies (24). The IE surface-exposed PfP2-protein occurs during a short window of erythrocytic development and the exported P2 protein appears to exist exclusively as an SDS-resistant P2-homotetramer (24). This indicates a developmental regulation of oligomerization of Plasmodium P2 protein.

The structure of the N-terminal dimerization domain of human P2 protein was determined recently by NMR (25). By homology modelling, a structural model of P1/P2 dimerization domain was proposed and this model predicted that helix-3 of P1 is not involved in P1/P2 dimerization, but plays an important role in the formation of P-complex (25). The hetero-oligomerization of P1/P2 proteins have been reported earlier, and these appear to be precursors to the pentameric ribosomal P-protein complex (26). However, P1 and P2 proteins have been found in the cytoplasmic pool, which exchange with those on the ribosome, and also form homo-oligomers (26). For P. falciparum P2 protein, so far NMR structure has been possible only under denaturing conditions, because the recombinant PfP2 protein undergoes extensive oligomerization at concentrations of about 1 mM (27).

Here we report the biophysical properties of the ribosomal P2 protein of P. falciparum, with special emphasis on oligomerization. We demonstrate that the 53rd cysteine residue of PfP2 is vital for SDS-resistant DTT-sensitive oligomerization, and that the P2 protein shows anomalous folding due to the acidic C-terminal domain. We also show that apart from the SDS-sensitive pentameric complex, the P2 protein...
forms developmentally regulated DTT- and SDS-resistant oligomers, and that certain differentially expressed protein components of the parasite appear to play important roles in such oligomer formation.

EXPERIMENTAL PROCEDURES

Ethics Statement - Tata Institute of Fundamental Research (TIFR) Animal House is registered under CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of environment and forest, Govt. of India (registration no. 56/1999/CPCSEA) for breeding and experiments on animals. This study was carried out under strict accordance with the guidelines of CPCSEA, India, for the care and use of laboratory animals. The study was approved by the institutional animal ethics committee, TIFR, Mumbai (Project no. TIFR/IAEC/2008-1) formulated by CPCSEA.

Human blood was collected from volunteers after obtaining their written consents, for the in-vitro cultures of Plasmodium falciparum. The procedure for such collection, details of informed consent and the frequency of samples to be collected were in accordance with a detailed proposal approved by the Institutional Human Ethics Committee (IHEC) of TIFR. The IHEC of TIFR is constituted as per the guidelines of Indian Council of Medical Research (ICMR), Govt. of India.

Cloning and expression of PfP2 (PFC0400w) gene and its mutants in pProExHTa vector - PfP2 gene was PCR amplified from Plasmodium falciparum (3D7) genomic DNA using the following primers containing EcoRI (New England BioLabs, NEB) at the 5' end and XhoI (NEB) at the 3' end restriction overhang, respectively.

Forward primer: 5'-CCCCGAATTCATGGCTATGAAATACGTTGCT G-3';
Reverse primer: 5'-GGGGCTCGAGTAAACCAAATAGGAAAATC GTAAGTCTC-3'.

Both the PCR amplified PfP2 gene fragment and the pProExHTa vector (Lablife) DNA were digested using EcoRI and XhoI restriction enzymes at 37°C, purified and ligated at 16°C for 16 hrs using T4 DNA ligase (Roche, Germany, cat no. 10481220001). DH5α competent cells were transformed by heat shock, and positive clones were identified through plasmid purification and restriction digestion. PfP2CA20 and PfP2CA40 were also cloned in the same vector following the same methodology, excepting that the amplifications were carried out using the following reverse primers:
PfP2CA20: 5'-GGGCTCGAGTTATTTCTTCTTCTTTTTCT TAG-3';
PfP2CA40: 5'-GGGGCTCGAGTTAACCACCCCTCAATTTTT G-3' keeping the forward primer as mentioned above.

GST-PfP1 construct was made by cloning PfP1 gene between EcoRI and XhoI sites in pGEX-4T-3 vector (GE Healthcare, USA). PfP1, with one postulated intron, was amplified from P. falciparum (3D7) cDNA library using the following primers:

Forward: 5'-CCCCGAATTCATGGCATCAATTCCAGCATC-3';
Reverse: 5'-GGGGCTCGAGACCAAATAAGGAGAAACC-3'.

The DNA (ORF) sequences of all the clones were confirmed by DNA sequencing. For generating mutants of PfP2, custom based site directed mutagenesis was carried out and five different mutant clones of PfP2 were generated, M1 (C12A), M2 (C53Y), M3 (C12A, C53Y), M4 (C53A) and M5 (C12A, C53A). The mutant genes were cloned in pProExHTa vector and nucleotide sequence was confirmed by DNA sequencing through Bangalore Genei, India.

Recombinant protein expression and purification - All constructs [P2, M1(C12A), M2 (C53Y), M3 (C12A, C53Y), M4 (C53A), M5 (C12A, C53A), P2CA20, P2CA40], were transformed in E. coli BL21 DE3 strain and protein expression was induced by 0.5 mM IPTG (Sigma-Aldrich, Inc, St. Louis, MO, USA, cat.I6758). However, M5 did not get expressed under similar conditions. Recombinant PfP2 (rPfP2), M1, M2, M3, M4, rPfpP2CA20 and rPfpP2CA40 proteins were purified using Ni-NTA beads (Qiagen, Hilden, cat. no. 30230). All the recombinant proteins, rPfpP2, M1, M2, M3, M4, rPfpP2CA20 and rPfpP2CA40, were fusion proteins containing additional 30 amino acids (aa) at the N-terminus, including 6-Histidine, totaling to 142 (for P2, M1, M2, M3, M4), 122 aa (for rPfpP2CA20) and 102 aa (rPfpP2CA40), respectively. PfP2 was cloned in pQE and pET vectors to obtain a non-fusion or a cleavable P2-recombinant protein. However, no stable
expression of PfP2 protein could be achieved without these additional 30 amino acids. PfP1 protein was expressed in BL21 cells as a GST fusion protein and purified using GST beads. 10 mM reduced glutathione was used to elute PfP1-GST protein from the beads.

**Parasite culture and synchronization** - *P. falciparum* 3D7 strain parasites were maintained in culture as described earlier (28). Human blood, from healthy adults with B* blood group, was collected in K2 EDTA vacutainers (BD Biosciences) as an anticoagulant. After removing the leukocytes, the erythrocytes were washed and resuspended in complete RPMI (cRPMI; RPMI with 0.5% Albumax). Asexual stages of *P. falciparum* 3D7 strain were cultured in vitro and maintained at 5% haematocrit in cRPMI at 37°C in a humidified chamber containing 5% CO₂. Synchronization of parasite was carried out using 5% sorbitol.

**Preparation of *P. falciparum* parasite, infected RBC cytosol and ghost proteins** - *P. falciparum* 3D7 infected RBCs at about 7-8% parasitemia was pelleted at 500 g for 5 min, and washed with cRPMI once. RBC pellet was resuspended in 0.1% saponin and protease inhibitor cocktail (Sigma-Aldrich, Inc, St. Louis, MO, USA, cat. P8340) and 1 mM PMSF in Phosphate buffered saline (PBS), pH 7.4 for 15 min at 37°C. Sample was then centrifuged for 10 min at 10,000 g at 4°C to get the parasite pellet, which was sonicated in lysis buffer (PBS, pH 7.4, with 0.1% Triton X-100 and protease inhibitors) to get the parasite protein and stored at -80°C. About 60–70% of the opaque supernatant (RBC ghost and cytosol fraction) was gently separated to avoid contamination from the parasite pellet. This supernatant fraction was pelleted at 20,000 g for 2 hrs at 4°C, washed twice with PBS, pH 7.4, and stored at -80°C for use as IE ghost. After ghost precipitation, the supernatant (IE cytosol) was stored at -80°C for subsequent analysis. All buffers contained protease inhibitor cocktail (Sigma-Aldrich, Inc, St. Louis, MO, USA, cat. P8340).

**Immunoblots of recombinant and parasite proteins** - The recombinant or parasite proteins were used for the immunoblots after protein estimation using Bradford reagent (Sigma). Before loading, the protein was mixed with gel loading buffer (50 mM Tris, Cl pH 6.8, 10 mM DTT/20mM β-Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 10 min. Samples were resolved on 12% SDS-PAGE and proteins were transferred to methanol-activated Polyvinylidene Fluoride (PVDF) membrane (Millipore) using anode buffer (25 mM Tris.Cl pH 10.4, 10% Methanol) and Trans Blot Semi Dry Transfer system (Bio-Rad, USA). Membranes were blocked with 5% non-fat skim milk powder in 1x PBS overnight and probed with specific antibodies. Primary antibody dilution was made in 1x PBS with 0.2% Tween (PBST) and incubated with the membrane for 3 hrs at room temperature (RT) on a rocker. Primary antibody binding was detected by appropriate secondary antibody conjugated to HRP (GE Healthcare, UK, cat no. NXA931). Dilution of secondary antibody was made in PBST. After every incubation, membrane was washed with PBST at least 6-7 times with 5 min interval. The immunoblots were developed using the ECL Plus TM (Amersham) as also by Super signal West pico chemiluminescent substrate (Thermo Scientific, USA). The antibodies used were anti-PfP2 monoclonal antibody E2G12 (1:100) (24) and anti-β-actin antibody (1:1000) (Sigma Aldrich, cat no. A1978).

**Mass spectrometry of recombinant PfP2 protein using MALDI-TOF** - Purified recombinant PfP2 protein was subjected to Mass Spectrometry to determine the molecular weight. For determination of mass, MALDI was used from Bruker Corporation (model no. 201344). The protein was dissolved in Tris Cl pH, 7.4 with 50 mM NaCl and reconstituted using 50% Acetonitrile and 0.1% TFA (500 µl of 100% acetonitrile + 499 µl of distilled water + 1 µl of TFA). The reconstituted protein was loaded on a MALDI plate using saturated solution of α-cyano hydroxyl Cinnamic acid (CHCA) (Bruker 201344) (~20 mg/ml) in 50% acetonitrile and 0.1% TFA. The proteins were ionized and molecular weight was determined. In Y axis the intensity of the protein in atomic unit (AU) and in the X axis m/z was plotted.

**Gel filtration of recombinant and parasite protein** - For gel filtration, the AKTA device from GE Healthcare was used which was coupled to UV spectrometer. The gel filtration profiles of all recombinant rPfP2, mutants, PfP2Δ20 and PfP2Δ40 proteins were determined using Superdex-75 column, using 1-2 mg protein. For parasite crude protein fractionation, 3-4 mg of parasite protein extract was injected in to Superdex-200 column. For each set of runs about
90 fractions were collected. The parameters of gel filtration device were; Pressure: 0.12 MPa, Column Bed Volume: 120 ml; Flow rate: 1 ml / min, Fraction collection rate: 1 ml/min; Empty loop volume: 40 ml. For runs under reduced conditions, 10 mM DTT was added. Fractions were concentrated to 20% volume and 10 µl concentrated samples from each fraction was resolved on 12% SDS-PAGE followed by immunoblot using anti-PfP2 monoclonal antibody E2G12 (24).

Interaction of P2 with gel filtration beads - Gel beads from different column materials (Superdex, Sephadex G50 and Biogel P60) were equilibrated in PBS and used as a 50% bead slurry for further binding experiments. 100µg of P2 (1 µg/µl) in PBS was incubated with 50 µl of a 50% bead slurry of Superdex, Sephadex and Biogel beads at room temperature with constant mixing for one hour. Additionally, some samples were incubated in PBS containing 500 mM NaCl. The beads were subsequently pelletted down by centrifugation at 1000 g for 5 minutes and the supernatant collected. The beads were then washed for the indicated number of times by resuspension in 20 bed volumes of PBS followed by centrifugation. The beads and post-bead supernatant were boiled with gel loading dye for 10 minutes before being resolved on a 12% SDS PAGE followed by staining with Coomassie brilliant blue R-250.

ConA was used as a positive control while incubating P2 with superdex 75 gel filtration beads. 100 µg of ConA was incubated with gel filtration beads. Post bead treatment and 15 washes, beads and post bead supernatant was resolved in 12% SDS-PAGE followed by Coomassie staining.

Circular Dichroism (CD) - Far-UV circular dichroism (CD) spectra of the protein at 25°C were recorded on a JASCO-J810 spectropolarimeter (Jasco, Hachioji, Japan) using 0.1cm cell. Spectra for P2 and point mutants as well as deletion constructs were recorded. The protein concentration was 30 μM. The CD machine was pre-calibrated with Iolar Nitrogen for 1 hr before starting. The samples at appropriate conditions were equilibrated for at least 10-12 hrs before CD measurements. Each spectrum was an average of three scans (slit width of 2 nm). The data was plotted as millidegree vs the UV wavelength (from 190 nm to 250 nm) in the two dimensional graph and compared with standards to estimate the extent of α helicity, β-sheet and random coil. Near-UV CD spectra were recorded using a 1 cm cell, with protein concentrations at 1.5-2 mg/ml. Each spectrum was an average of ten scans (slit width of 2 nm) and the data was plotted as the CD in millidegree vs the UV wavelength (from 250 to 320 nm).

Fluorescence labeling of recombinant PfP2 and PfP1-GST proteins with tetramethyl-rhodamine (TRITC) - About 2 mg of recombinant PfP2 protein was buffer exchanged with 1 ml of 0.1 M sodium bicarbonate buffer (pH 8.5). While labeling the protein, the molar ratio of protein vs dye was 1:10 (10 fold more dye was used). 5 mg of tetramethyl rhodamine (Invitrogen) was dissolved in 0.5 ml of DMSO (Dimethylsulfoxide). The labeling of protein was done according to the manufacturer protocol (Invitrogen). The final concentration of protein was 0.5 mg/ ml. While stirring the protein solution, slowly 50–100 µl of dye solution was added. Protein and dye solution was incubated at room temperature for 1 hr. 1.5 M hydroxylamine, pH 8.5, was added into the protein dye solution to terminate the reaction. 3 kDa Membrane filtration cut off device was used to remove the unbound dye and to purify labeled protein. Recombinant GST-PfP1 protein was also conjugated with tetramethyl-rhodamine using the same protocol.

Treatment of tetramethyl rhodamine labeled rPfP2 protein with parasite lysate and FPLC fractions - Synchronized P. falciparum cultures were harvested at different time points post merozoite invasion (PMI), and parasite lysates were prepared. Parasite lysate was centrifuged at 12000 g for 1 hr at 4°C and supernatant was collected. Supernatant protein was quantified using Bradford or BCA. About 5 µg each of parasite crude extract from different time points (PMI) was incubated with 1 ng of tetramethyl rhodamine labeled rPfP2 at 37°C for 3 hrs in water bath with intermittent mixing of the solution. Post-incubation, the mixture was boiled with SDS-loading buffer containing β-Mercaptoetanol and resolved on a 12% SDS-PAGE. The gel was scanned using Typhoon TRIO (GE Healthcare) to check the presence of SDS resistant fluorescent protein oligomer bands. The PMT gain of the scanner was set at 250 volt. The λex of tetramethyl rhodamine was 532 nm and λem was 580 nm.

For the FPLC fractions, 25 µl of the pooled fractions were used for incubation of 1 ng
of tetramethyl rhodamine labeled rPfP2 or GST-PfP1 protein at 37°C for 3 hrs, and monitored for SDS resistant fluorescent protein band on SDS-PAGE as described above.

RESULTS

SDS-resistant oligomerization of rPfP2 and its mutants - The Plasmodium ribosomal protein P2 displays a novel non-ribosomal role, as also exhibits oligomerization at the trophozoite stages in the erythrocytic development cycle (24). The recombinant PfP2 (rPfP2) protein also exhibits oligomerization (24, 27). To evaluate the propensity of SDS-resistant and -sensitive oligomer formation, the recombinant protein rPfP2 and certain recombinant PfP2 mutant and deletion proteins (Figure S1) were run on SDS-PAGE and through gel-filtration columns (Figures 1 and 2).

Coomassie stained SDS-PAGE of rPfP2 protein showed the monomer along with large amounts of SDS-resistant dimers (Figure 1A, Lane 1), which were verified as PfP2 dimers (Figure 1B, Lane 1) using PfP2 specific mAb E2G12 (24) To check the role of disulfide bonding in such dimer formation, rPfP2 was run in the presence of the reducing agent DTT. The dimers were found to abolish in the presence of reducing agent (Figure 1A, 1B Lane 2). In P. falciparum, two cysteine residues are found in the P2 protein at 12th and 53rd positions for the 3D7 strain (Figure S2-4). These were confirmed by sequencing the rPfP2 clone, prepared from 3D7 strain genomic DNA. The sequence was identical in six different Indian isolates, as also in different laboratory strains of P. falciparum (H. Joshi and S. Sharma, unpublished data). Interestingly, although the 12th cysteine is conserved in all Plasmodium species, the 53rd cysteine is not present in the rodent species of Plasmodium, namely P. berghei, P. yoelii and P. chabaudi (Figure S4). Conversely, P. falciparum does not possess a tyrosine residue conserved at the 53rd position in the rodent and simian parasites. However, the tyrosine residue is also absent in the malaria parasite species of avian, P. gallinaceum, and in the chimpanzee, P. reichenowi (Figure S4).

To test the contributions of the two cysteines of P. falciparum P2 protein towards dimerization, several different recombinant clones, M1 (C12A), M2 (C53Y) and M3 (C12A, C53Y) were generated (Figure S1). The C53Y mutation for M2 protein was guided by the observation that a tyrosine residue was found in certain species of Plasmodium P2 proteins at the 53rd position (Figure S3, S4). The recombinant P2 protein from M1 clone showed that the replacement of the C12 residue had no significant effect on the DTT-sensitive rPfP2 dimer amounts, and behaved in a fashion similar to rPfP2 protein (Figure 1A, B). M2 protein, with a C53T mutation, showed a large decline in dimerization. M3 protein, with the double mutations, behaved similar to the M2 mutant (Figure 1 A, B). In order to rule out an influence of the tyrosine residue at the 53rd position, constructs M4 (C53A) and M5 (C12A, C53A) were also generated, shown schematically in Figure S1. M4 behaved the same way as M2, and exhibited no detectable dimer on Coomassie stained SDS-PAGE (Figure S5A). Mutant M5 did not show adequate protein expression (Figure S5B), and therefore it was not possible to assess how the double mutant (C12A, C53A) of PfP2 would behave. However, from the results using M4, it could be deduced that the C53 residue played a dominant role in the formation of SDS-resistant, DTT-sensitive dimers. However, the continued presence of small amounts of SDS- and DTT-resistant oligomers in rPfP2 indicated that although rPfP2 oligomerization was influenced by disulfide bonding, other interactions were also operative.

The deduced molecular mass of the parasite PfP2 protein is 12 kDa. The recombinant rPfP2 protein contains additional 30 amino acids (from the vector pProExHTa) and the monomer and dimers correctly showed the expected mass of 15,884 and 31,336 kDa on MALDI mass spectrometry (Figure S6). However, on SDS-PAGE, the monomeric and dimeric rPfP2, as also the PfP2 protein from parasite extracts, migrated at higher molecular weights of 18 and 36 kDa; and 16 and 32 kDa, respectively (Figure 1A-D). Since the C-terminal region is negatively charged, it is possible that this repels the negative charge of SDS and hinders SDS binding. When SDS binding is less, mobility would be less, giving an anomalously high molecular weight. In silico prediction (DisEMBL 1.5) shows that amino acids from 72-112 of PfP2 protein are disordered in nature, and therefore this abnormality could also be due to this disordered acidic C-terminal region not conforming to globular structure, resulting in abnormal migration on SDS-PAGE. To test the contribution of the acidic C-terminal domain on oligomerization and mobility on SDS-PAGE, two...
Developmentally regulated oligomerization of Plasmodium P2 protein

deletion constructs, rPfP2Δ20 and rPfP2Δ40 proteins, with C-terminal 20 aa and 40 aa deletions respectively, were expressed, and run on SDS-PAGE (Figure 1A, B). While rPfP2Δ20 (deduced molecular mass 12 kDa) was still migrating abnormally, rPfP2Δ40 (deduced molecular mass 10.6 kDa) behaved normally and migrated at the expected size of 11kDa on SDS-PAGE (Figure 1A, B). Once again, the dimers of these deletion proteins were largely DTT-sensitive. However, significant amounts of SDS- and DTT- resistant oligomerization continued to occur in the rPfP2Δ20 and rPfP2Δ40 proteins (Figure1B). Indeed, the extent of oligomerization seemed to be slightly enhanced in the rPfP2Δ40 protein (Figure 1B). Thus the acidic C-terminal region hindered the normal migration of PfP2 protein on SDS-PAGE and the DTT-sensitive dimerization was crucially dependent on C53 residue. As expected, the DTT- resistant oligomerization was independent of both the cysteine residues.

The concentration of P2 protein in Plasmodium cells is around 20-50 nM, as determined through ELISA assays using rPfP2 as a standard. In the test tube, rPfP2 does not form SDS resistant oligomers at that concentration, but were detected in the parasite extracts even at these low concentrations (Figure 1C, D). An analysis of different developmental stages of P. falciparum extract showed that SDS-resistant, DTT-sensitive oligomers (mainly dimers) were detected throughout the erythrocytic stages, but at certain specific stages, 24 to 36 hrs post-merozoite invasion (PMI), SDS and DTT-resistant oligomers were produced (Figure 1C, D). In summary, PfP2 protein readily formed dimers that are SDS-resistant but largely DTT-sensitive. However, a fraction of the SDS-resistant oligomers are DTT-resistant, and these are developmentally regulated.

Multiple bands were seen in the native PAGE for both the recombinant and parasite PfP2 proteins (Figure 1E), as seen through Coomassie staining and upon probing with anti-PfP2 mAb E2G12 (24). We were unable to obtain an NMR solution structure of rPfP2 since this protein formed aggregates at the concentration required for a solution NMR structure (> 1 mM) (27). The formation of DTT- and SDS-resistant rPfP2 dimers and higher oligomers was concentration dependent and in vitro the oligomers were observed at > 0.1 µg/µl (about 10 µM) concentration (Figure 1F).

Thus, both rPfP2 in vitro and PfP2 in vivo exhibited SDS-resistant oligomers. While a large part of the DTT-sensitive forms were dimers, it was the DTT-resistant oligomers that exhibited developmentally regulated formation in the parasite.

**Gel filtration profile of rPfP2, M1, M2, M3, rPfP2Δ20 and rPfP2Δ40 proteins** - All of the above data pertained to SDS-resistant oligomerization properties of PfP2 protein. To assess the nature of oligomeric protein complexes present in rPfP2 solution, 1 mg of purified rPfP2 and mutant proteins were run on a 120 ml Superdex-75 gel filtration columns under reducing and non-reducing conditions (Figure 2A, B). Several molecular markers were run to calibrate the column. In the presence of DTT, each of the wild type rPfP2, M1 and M2 proteins eluted as a single peak at 120 ml fraction volume, projecting at a globular size far smaller than aprotinin (6.5 kDa) which eluted at 90 ml fraction volume (Figure 2A). However, M3 protein behaved differently and some amount of recombinant protein was eluted at 60 ml fraction volume (75 kDa) while a larger proportion eluted at the same 120 ml fraction volume (Figure 2A). In the absence of DTT, rPfP2 showed a peak that mapped to 120 kDa, and a minor peak at 75 kDa, while M3 protein showed predominantly 75 kDa and large aggregates that eluted in the void volume (> 250 kDa) (Figure 2B I, II). Thus both the rPfP2 protein and the double mutant aggregated to various molecular sizes under non-reducing conditions. The Peak 1 continued to occur in both rPfP2 and in the double cysteine mutant M3 under non-reducing conditions as well (Figure 2B).

Gel filtration column separates proteins by their hydrodynamic volumes and for sets of proteins with similar conformation; the hydrodynamic volume is proportional to the molecular mass. The columns were calibrated with mainly globular proteins. If the rPfP2 is not a globular protein, its chromatographic behaviour will change. Moreover, an interaction of rPfP2 with the Superdex column material may also retard the movement of the protein through the column. The proteins may be degraded so that they migrate abnormally. Coomassie staining showed the presence of intact 18 kDa rPfP2/mutant proteins in the Peak fractions, with no evidence of degradation (Figure 2 CI). The other fractions were also run on SDS-PAGE and only the peak fractions at 120 ml showed the presence of...
Developmentally regulated oligomerization of Plasmodium P2 protein

recombinant PfP2 proteins. To test the possibility of an interaction of rPfP2 with the gel filtration column material, rPfP2 was incubated with Superdex-75 beads, and the beads were assessed for bound rPfP2 protein (Figure 2CII). It was observed that rPfP2 protein does bind weakly to Superdex-75 gel filtration materials. The binding is weak and with multiple washes, the bound protein amount reduces (Figure 2CII). Similar behaviour was seen with Sephadex G-50 and Biogel beads (Figure S7I). The presence of NaCl in the binding buffers did not make much difference to this weak binding (Figure S7II). With extensive washings the rPfP2 protein showed no signature of binding, although the sugar binding protein Concanavalin-A did remain bound to the beads as expected (Figure S7III). Therefore the abnormal migration of the rPfP2 and the mutants could be partly due to weak non-specific interactions with the column matrix.

To check the effect of the disordered acidic C-terminal region on the abnormal migration of rPfP2 on gel-filtration, the two deletion constructs rPfP2Δ20 and rPfP2CA40 proteins were run on FPLC (Figure 2D). It was observed that the deletions did influence the abnormal migration of rPfP2, and the major amount of protein were eluted at the expected monomeric size of 11.0 kDa for the rPfP2CA40 protein (Figure 2D). A peak was still observed at 120 ml fraction volume, but no intact rPfP2CA40 protein was observed on SDS-PAGE (Figure 2D), indicating a possible lack of stability in the rPfP2CA40 protein and collection of very small degradation products. Thus, the anomalous gel filtration behavior appears to be mainly due to shape aberrations and non-specific interactions caused by the highly negatively charged C-terminal region. Eliminating this region resulted in an expected elution profile for a globular protein.

Circular dichroism (CD) measurements of rPfP2 and the various mutant/deletion proteins - We have reported CD data of rPfP2 protein and the rPfP2CA40 earlier (27). A CD study of rPfP2 protein at the far-UV region shows features of largely alpha-helical structures, both in the presence and absence of reducing agent (Figure 3A, B). Comparison of the CD spectra of the P2 proteins under reducing and non-reducing conditions, as well as amongst different mutants, did not show any significant differences. As shown earlier (27), the CD spectra predicted about 30% α-helicity. CD spectra of both the deletion constructs, rPfP2CA20 and rPfP2CA40, also showed the canonical deeps at 220nm and 208nm (Figure 3C, D), indicating the alpha helical nature of both the deletion proteins. The extent of random coil was reduced considerably in the deletion proteins, which indicated that the carboxy-terminal amino acids did contribute to the disordered random coil structures in the PfP2 protein. In cases of both rPfP2CA20 and rPfP2CA40, the negative millidegree increased, which indicated that with the deletion of the acidic C-terminus, the α-helicity of the proteins increased.

To assess the tertiary features of P2 protein, near-UV CD data were recorded (Figure 3E, F). The data indicates a lack of significant tertiary structure in each of the proteins rPfP2 and the mutants. However, as expected, the lack of disordered regions amongst the deletion proteins rPfP2CA20 and rPfP2CA40, gave rise to some tertiary structure. It was interesting to note that the deletion of the penultimate 20 aa (71 to 91 residues), containing large number of alanine residues, reduced the extent of tertiary structure, as seen with the CD data of rPfP2CA40 protein. Through NMR analysis, we have demonstrated earlier that both the rPfP2 and the rPfP2CA40 proteins form large aggregates (27). Despite such inter-molecular associations in solution, the near-UV CD data showed the absence of significant tertiary structural features in both rPfP2 and rPfP2CA40.

Gel filtration profile of P. falciparum parasite protein - To observe the state of the PfP2 complexes in the parasite, total parasite lysates were run on FPLC and the fractions were tested for the presence of various oligomers of PfP2. Since we were aware that critical changes in SDS- and DTT-resistant oligomerization occur around 24 hrs PMI, elution profiles of parasite lysate at 18 and 30 hrs PMI were assessed on a Superdex 200 column in the presence of DTT under reduced conditions (Figure 4A, C). Fractions of 1ml were collected, and a standard curve was generated using several molecular weight standards. The presence of PfP2 protein was determined by running every fraction on SDS-PAGE under reducing conditions and probing with PfP2-specific monoclonal antibody E2G12 (24). No PfP2 molecules were detected at fractions corresponding to 16 kDa and 32 kDa sizes, indicating that there was not a significant presence
of free PfP2 monomers or dimers at either of these stages. A peak of PfP2 protein was noted around fractions 76/77 (about 90-100 kDa) in both the stages (Figure 4). The ribosomal pentameric complex of P0-2P1-2P2 calculate to 90 kDa theoretically, with molecular weights of P0 as 38 kDa, P1 as 13 kDa and P2 as 12 kDa. However, all of the three \textit{P. falciparum} proteins migrate at higher molecular weights in SDS-PAGE (24) and exhibit sizes of PfP0:35-37 kDa; P1: 17-18 kDa and P2:16 kDa, respectively. This would estimate a size of about 90-105 kDa for the pentameric complex and the peak at fractions 76/77 would be consistent with this size. No other molecular species of PfP2 was detected at the 18 hrs PMI. The PfP2 present in this complex resolved entirely as a monomeric 16 kDa PfP2 band on SDS-PAGE, showing the absence of detergent-resistant oligomers at 18 hrs PMI.

In contrast, a dominant presence of additional large aggregates (>600 kDa) were noted during the 30 hrs stage (Figure 4 C, D). These large aggregates also resolved into PfP2 monomers and did not show any other SDS-resistant species. However, small amounts of PfP2 complexes, observed at fraction numbers from 97-102 from 84-85 and 88-91 were detected in the 30 hrs PMI sample (Figure 4D) mapping to average molecular sizes of 29 kDa and 60-80 kDa respectively (as determined through markers on the gel-filtration column). The 29 kDa fractions would map to dimeric PfP2 (32 kDa) protein, and the 60-80 kDa fractions would correspond to tetrameric 64 kDa species on SDS-PAGE. Thus at 30 hrs PMI under reducing conditions, the parasite appeared to contain no native free monomer of PfP2, small quantities of dimers and tetramers of PfP2 which were SDS-resistant, and predominantly the pentameric P-protein complex and large aggregates, which were SDS-sensitive (Figure 4D).

A mild saponin treatment of infected RBCs (iRBCs) typically yields a parasite pellet, iRBC cytosol and iRBC ghost membranes. The 30 hrs iRBC cytosol and ghost membrane fractions were resolved on FPLC and the fractions were probed for PfP2 protein (Figure 5 A-D). Neither of these extracts exhibited the prominent 76/77 peak of PfP2 which presumably represents the pentameric ribosomal P-complex. Nor did either of the extracts exhibit any monomeric P2 protein on SDS-PAGE. This is consistent with our earlier observations of lack of monomeric PfP2 proteins in immunoblots of iRBC ghost and cytosolic preparations (24). The iRBC cytosol and the iRBC membrane fractions from 30 hrs PMI parasite preparation showed the presence of only a 64 kDa PfP2 band on immunoblot. In the iRBC cytosol, this SDS-resistant tetrameric species occurred in gel fractions corresponding to molecular complexes ranging from 50 to 80 kDa, as also in the large aggregate complexes with a size > 600 kDa that eluted with the void volume (Figure 5B). However, the iRBC ghost membrane fractions did not show any large complexes but showed the PfP2 species exclusively at ranges of 50 to 70 kDa (Figure 5D). Mass spectrometry analysis of this band shows predominantly peptides of P2 protein and no other peptide with significant score (24). No PfP2 bands could be detected in any fractions when the 18 hrs iRBC cytosol and ghost membrane samples were run through gel-filtration columns (data not shown).

These results showed that large aggregate complexes containing PfP2 occur in the parasite as also in the red cell cytoplasm at 30 hrs PMI. Only the SDS-resistant tetramers move out through the red cell cytoplasm as a large complexes. At the iRBC membrane, PfP2 is detected exclusively as 60-80 kDa size species, possibly representing the tetrameric PfP2. On SDS-PAGE both the RBC cytosol and membrane resolved to a uniform 64 kDa SDS-resistant species. All of the above studies were carried out under reducing conditions and therefore represent DTT-resistant PfP2 species.

\textbf{Effect of parasite extracts on oligomerization of rPfP2} - The erythrocytic stages of \textit{P. falciparum} cycles is about 48 hrs, moving through ring, trophozoite and schizont stages (Figure 6A), within which SDS- and DTT-resistant oligomers of PfP2 were produced in the parasites beyond 24 hrs PMI (Figure 1,4,5). To test if certain protein components of the parasite were promoting such oligomerization, the rPfP2 protein was treated with total parasite lysates at different PMI stages and run on SDS-PAGE (Figure 6B). The rPfP2 was labeled with tetramethyl-rhodamine, so that selectively the changes in rPfP2 protein could be tracked through the fluorescence. The reaction was carried out at 1 ng/µl concentration of labeled rPfP2, where no self-association of rPfP2 would be observed (Figure 1F). It was observed that 24 and 30 hrs parasite lysates could indeed propel
Developmentally regulated oligomerization of Plasmodium P2 protein

aggregation, while 6, 12, 18 and 42 hrs extracts were not so effective in such oligomer formation (Figure 6B). In order to dissect out *P. falciparum* proteins responsible for such oligomerizations, pools of 5 FPLC fractions from 30 and 18 hrs PMI samples (Figure 4) were used for the treatment of rhodamine-labeled rPfP2 and such incubated fractions were run on SDS-PAGE (Figure 6 C, D). While none of the 18 hrs samples exhibited significant oligomerization (Figure 6D), certain pools of proteins from 30 hrs PMI parasite sample caused distinct rPfP2 oligomerizations (Figure 6C). The oligomerization was observed upon incubation with mainly fractions 65-69 (molecular size 230 to 183 kDa) and 80-84 (molecular size 85 to 70 kDa). However, when individual fractions from these pools were tested, no oligomerization was observed. A representative data of treatment with individual fraction numbers 80-84 are shown in Figure 6E. The effect was specific to PfP2, since rhodamine labeled PfP1-GST protein did not exhibit such oligomer formation (Figure 6F). The pooled fractions 80-84 always showed oligomer formation and was used typically as a positive control (Figure 6 D, F, G). The properties of the effective pooled fractions were likely to be due to the protein components, because heat treatment abolished such oligomerizing capability (Figure 6G). Thus specific protein components of 24-30 hrs PMI parasite appear to propel the oligomerization of PfP2 as seen through our rhodamine-labeled rPfP2 assay.

DISCUSSION

In this paper we have analyzed the biophysical properties of the *P. falciparum* acidic ribosomal protein PfP2. The secondary structure of rPfP2 is largely α-helical. The extensive oligomerization states of the recombinant and the parasite PfP2 proteins have been detailed. The PfP2 protein shows SDS-resistant oligomers that are sensitive to reducing conditions. The 53rd cysteine residue, but not the 12th cysteine residue, appears to be mainly responsible for such DTT-sensitive, SDS-resistant dimerization. The acidic disordered C-terminal 40 aa residues contributed towards abnormal migration of the recombinant protein in the gel filtration column. The PfP2 protein in the parasite lysate was largely associated with the pentameric ribosomal P-protein complex, at more or less the expected size and did not exhibit the retarded behaviour shown by the recombinant protein on gel filtration column. A lack of free C-terminal acidic end of the PfP2 protein within the parasite extract would explain this difference in migration of the rPfP2 and the parasite PfP2 protein on gel filtration chromatography. Apart from the ribosomal P-protein complex, larger complexes containing PfP2 protein were observed in 30 hrs PMI samples, and these complexes resolved to DTT- and SDS-resistant tetrameric structures in SDS-PAGE. Rhodamine labeled rPfP2 protein was used for monitoring oligomer formation on SDS-PAGE. Using this simple visual technique, it was demonstrated that multiple parasite protein components are apparently involved in the stage-specific formation of PfP2 oligomers.

Progressively it is becoming clear that several proteins function as complexes in the cell. Amongst these, the proportion that form self-complexes (or with very similar peptide chains) are considerable (29). An estimate of such homooligomeric proteins have indicated that there are significantly larger number of self-interacting proteins than would be expected randomly (30, 31). Apart from general folding and stability issues, such a form of generating new protein surfaces using a single peptide chain would provide the cell with new functions and regulation without additional burden on gene pool. Facilitating such oligomerization with development specific protein(s) could regulate new activities, trafficking and pathways.

Disulfide bond and PfP2 protein oligomerization:
The role of disulfide bonding in protein oligomerization is well established. The importance of cysteine residues in such a process, with consequences to protein functions, has been demonstrated in numerous experiments using chemical modification techniques and the cysteine scanning mutagenesis (32, 33). Several malaria vaccine candidates’ surface antigens, such as MSP1, AMA1 and EBA175, possess cysteine rich motifs, which are engaged in disulfide bonding. Critical conformation domains belonging to these proteins are sensitive to reducing conditions (34-36). It is curious that the P2 protein of most organisms, other than a few protozoans, do not possess any cysteine residues (Figure S3). The Apicomplexan species closest to *Plasmodium*; *Toxoplasma* and *Babesia*, possess no cysteine residues (Figure S3). Thus it would be expected
that disulfide bonding or reducing conditions would play no roles in the structural versatility of these P2 proteins. Several of the protozoan P2 proteins such as those from *E. histolytica, T. annulata* and *L. mansoni* contains a single cysteine residue within 12 to 28 aa positions (Figure S3). Rodent malarial parasites, *P. yoelii, P. berghei* and *P. chabaudi*, also possess only one cysteine residue at the 12th position (Figure S4). This C12 is conserved in all *Plasmodium* P2 proteins, but did not appear to play a role in the SDS-resistant DTT-sensitive oligomerization. The cysteine residue around position 53 is seen in all *Plasmodium* species, other than the rodent species (Figure S4). It occurs at the 52nd position in the related simian/human malaria *P. vivax* and *P. knowlesi*, at 54th position in avian *P. gallinaceum*, and at 53rd position in *P. falciparum* and *P. reichenowi*. The chimpanzee malaria parasite *P. reichenowi* is phylogenetically closest to the human parasite *P. falciparum* (37), and hence the similarity in the groups is understandable. Interestingly the apparently conserved tyrosine residue at 53rd position in the rodent and simian parasites is not observed in the other species (Figure S3, S4).

From the results presented in this report it is apparent that the conserved cysteine at the 12th position does not seem to play a role in the DTT-sensitive oligomerization in *Plasmodium*. On the other hand, the 53rd cysteine residue in *P. falciparum* is important for such DTT-sensitive oligomers (largely dimers). The functions of neither the DTT-sensitive dimers nor DTT-resistant oligomers of PfP2 in the parasite is clear at present. The DTT-sensitive dimers exist in *P. falciparum* throughout all developmental stages, but it is the DTT-resistant oligomers, that seem to occur in a development dependent fashion. Not only are these oligomers generated at a certain stages of the parasite (24-30 hrs PMI), some of these oligomers get exported through the red cell cytosol to the erythrocyte membrane (24). The erythrocyte membrane at 30 hrs PMI showed the presence of 60-80 kDa species that eluted from the gel-filtration column, but these resolved to a single 65 kDa SDS- and DTT-resistant PfP2 on SDS-PAGE (Figure 5). In contrast, the predominant species of PfP2 protein in the parasite protein preparation consisted of large aggregates (>600 kDa) and the pentameric complex, which yielded monomeric PfP2 on SDS-PAGE (Figure 4). Small quantities of SDS- and DTT-resistant dimer and tetramers were also detected in the parasite lysate. The infected-RBC ghost membrane showed no large aggregates but only the 60-80 kDa species which resolved exclusively to the SDS- and DTT-resistant tetrameric component and no monomer. However, it was interesting to note that the infected-RBC cytosol contained several complexes, one set around >600 kDa, and another set ranging from 60-80 kDa. All of these complexes resolved to 65 kDa SDS- and DTT-resistant PfP2 on SDS-PAGE, and no monomer was detected (Figure 5). As expected, the pentameric complex was limited to the parasite, while the species transported to the RBC cytosol and membrane belonged to different complexes. This is consistent with the ribosomal role of the pentameric complex. It is also apparent that the translocation through the RBC cytosol was occurring through a range of molecular complexes, with mainly the smaller 60-80 kDa complex localized to the RBC membrane. As far as the SDS- and DTT-resistant components are concerned, within the parasite largely monomeric species existed (with small quantities of dimeric and tetrameric species), while the IE-cytosol and ghost showed the exclusive presence of SDS- and DTT-resistant 65 kDa tetrameric component.

We have earlier shown that the SDS- and DTT-resistant 65 kDa band consisted exclusively of the PfP2 peptides, analyzed through immunoprecipitation and mass spectrometry analysis (24). It has also been shown that the PfP2 is exposed on the infected red cell surface and that blocking of PfP2 with specific monoclonal antibodies arrested the parasite at the onset of cell division (24). With the demonstration of various PfP2 oligomeric complexes in the IE-cytosol (the present study), an analysis of the composition of such PfP2 complexes in the IE-cytosol may help to elucidate the mechanism of translocation of PfP2 protein to the red cell surface.

**Regulation of SDS-resistant oligomer formation:**

Oligomerization and aggregation of proteins occur extensively in nature (29). The pathological state of various neurological disorders is associated with the accumulation of insoluble amyloid fibrils. In Alzheimer’s disease these amyloid fibrils are formed by multimerization of 39-42 aa residues amyloid peptide (Aβ) (38, 39). Many types of oligomeric amyloid-β assemblies have been described (40). It has been reported that Aβ2 forms
The PfP2 protein has mainly alpha-helical structures, whereas the amyloid-folding proteins are characterized by the beta-sheet structure of the peptides. However, this applies mainly to the peptide regions of amyloid proteins that are directly involved in the formation of amyloid axis. For beta-sheet structures it has been postulated that hydrophobic and aromatic residues influence peptide self-assembly (58, 59). Apart from intrinsic properties, self-associative structures are influenced by modulators such as metal ions (60, 61) and drugs such as curcumin or chloroquine (62, 63). Interestingly, the disordered N-terminal octapeptide region of mammalian prion protein PrP, which is not essential for prion infectivity, plays a major role in the self-association of the prion protein (64, 65). Although the PfP2 protein has a strong disordered region at the C-terminal end, in the del40 recombinant PfP2 the DTT-and SDS-resistant oligomerization is seen to persist, suggesting that the acidic C-terminal disordered region is not an absolute necessity for oligomerization. Recent examinations of the structural properties of amyloid fibrils formed spontaneously by recombinant mammalian PrP indicated a major refolding of the C-terminal domain from alpha-helix to beta-structure (66, 67). Such a possibility of the DTT-and SDS-resistant oligomeric structure arising from such a conversion of alpha-helix rich PfP2 to a beta-structure, therefore, may exist. Indeed, an NMR assessment of the PfP2 protein in the denatured state indicated a beta-structure propensity on the basis of secondary chemical shift analysis (27). Thus, although the PfP2 protein primary sequence appears to be quite distinct from the amyloid proteins, the possibility remains that PfP2 may represent an amyloid species. The determination of the structure of the PfP2 oligomers will help to resolve such a possibility.

Distinct states of oligomerization of substrate proteins resulting in differential trafficking have also been documented. For instance, angiopoietin 2 modulates the endothelial receptor tyrosine kinase Tie2 and induces Tie2 translocation to the specific cell-matrix contact sites located at the distal end of focal adhesions. It is reported that the different oligomeric/multimeric forms of the angiopoietins cause induction of distinct patterns of Tie2 trafficking (68). Similarly phosphorylation and dimerization regulate nucleocytoplasmic shuttling of mammalian proteins.
Developmentally regulated oligomerization of Plasmodium P2 protein

STE20-like kinase (69). Such post-translational modifications of proteins, resulting into multiple states of oligomerization that lead to differential functions/trafficking, have been extensively documented (29). The P2 protein has been shown to be phosphorylated in mammals and yeast at the serine residues at the carboxy-terminal end (70). PfP2 does not possess these serine residues, but there is a conserved C-terminal serine residue at 109 position (Figure S4). However, the observation that the deletion of C-terminal 40 amino acids does not affect SDS- and DTT-resistant PfP2 oligomerization indicates that this serine may not be important for such an oligomerization. *P. falciparum* possesses other serine/threonine aa at positions 21/22 and 52, but these are not conserved in the rodent malarial parasite (Figure S4). Since we see definite oligomerization in *P. berghei* and *P. yoelii* parasites, these aa residues are unlikely to be crucial. The two serine/threonine residues at 47th and 58th positions, and a tyrosine at position 9 are conserved throughout *Plasmodium*, and these may be sites for possible phosphorylation. Our observations that rPfP2 oligomerization occurs without any added source of ATP (Figure 6) indicates that such an *in vitro* process precludes active kinase reaction(s). It does not exclude possibilities such as nucleation of the complex with phosphorylated P2 protein present in those appropriate fractions, or the process of dephosphorylation of PfP2 through phosphatase(s). Mass spectrometry data on stage-specific parasite PfP2 protein will be informative regarding the post-translational modifications present at various positions in PfP2.

Most common method of analysis for assessment of SDS-resistant oligomerization is through immunoblotting using peptide specific antibodies (71, 72), as was used in our studies of PfP2 as well. However, the labelling of rPfP2 with rhodamine allowed us an easy visual assay for the activation of formation of SDS-resistant oligomers (Figure 6). Rhodamine labeling of peptides is an old and established technique, and such labeled proteins are typically assessed for their solution structures. However, in our case, we have used this simple method to search for parasite factors that may modulate SDS-resistant oligomerization. Since SDS-resistant oligomerization of peptides is a potential problem in various disease pathologies, the cellular factors responsible for such oligomerization may be accessed using this simple method.

Such a technique, using rhodamine labeled rPfP2 incubated with various parasite extracts, confirmed that there were developmental stage dependent factors in Plasmodium that propagated oligomerization of rPfP2 *in vitro*. Further, using gel filtration fractionated parasite proteins at the appropriate stage; we showed that a combination of multiple parasite protein components is required to promote oligomerization. Oligomerization of rPfP2 is a concentration dependent phenomenon and does not seem to depend on any other extrinsic factors *in vitro* (Figure 1F); indicating that the intrinsic structure of PfP2 molecule has the propensity to form oligomers. However, at very low concentrations, the oligomerization of rPfP2 is propelled through the presence of a combination of certain parasite proteins belonging to specific developmental stages (Figure 6). This happens without any added source of ATP, once again pointing to the non-covalent nature of aggregation. Analysis of the post-translational modifications on PfP2 and further dissection of parasite proteins to identify specific components that promote oligomerization will allow further understanding of the mechanism of developmental regulation of *Plasmodium* P2 protein oligomerization, and the export of the P2 protein through the infected red cell to its surface.
REFERENCES


Acknowledgements We thank Shashidhar Dolas and Dr. Rukmini Govekar from Advanced Centre for Treatment, Research and Education in Cancer (ACTREC, Mumbai, India) for helping us with MALDI. We also thank Reshma Korde for parasite cultures. We are grateful to A.S.R Koti for his comments on the manuscript. We are grateful to Dr. K Rajeshwari (Bioklone Pvt Ltd., India) for their excellent monoclonal antibody services.

Author Contributions S. Das and S. Rajagopal performed all the experiments and assembled all figures. S Das, S. Rajagopal and S. Sharma analyzed the data. S. Das and S. Sharma wrote the manuscript. S. Sivakami took part in critical discussions.

Funding This work was supported by an intramural grant from Tata Institute of Fundamental Research, DAE, India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Financial Interest
The authors declare no competing financial interests.

Authors Information
Correspondence and requests for materials should be addressed to SS (sharma@tifr.res.in)

Authors Declaration
Part of this work was presented by S. Das in Gordon Research Conference on Malaria,
Figure 1. P2 oligomerization of rPfP2 as well as in the *Plasmodium* parasite. A. Coomassie stained gel of 2 µg protein each of recombinant purified PfP2, PfP2 mutants M1 (C12A), M2(C53Y), M3(C12A,C53Y), PfP2 deletions P2del20, and P2del40 (Figure S1) proteins were resolved on 12% SDS-PAGE in the absence and presence of reducing agent (10 mM DTT). B. The same gel A was immunoblotted and probed with anti-PfP2 specific monoclonal antibody (E2G12) (24). C. About 4 µg each of parasite protein extracts obtained from synchronized *P. falciparum* cultures harvested at 18 hrs, 24 hrs, 30 hrs and 36 hrs post merozoite invasion, were separated on 12% SDS-PAGE in the presence of reducing agent (10 mM DTT), and the immunoblot was probed using anti-PfP2 monoclonal antibody E2G12 (24). D. 4 µg of crude *P. falciparum* parasite protein harvested at 18 hrs, 24 hrs, 42 hrs post merozoite invasion were separated on 12% SDS-PAGE without and with reducing agent 10 mM DTT and the immunoblot was probed using anti-PfP2 monoclonal antibody E2G12 (24). β actin was used as a loading control. E. I Coomassie stain of rPfP2 and II Immunoblot of 10 µg of asynchronous *P. falciparum* parasite crude protein extract, run on 10% native PAGE and probed using anti-PfP2 monoclonal antibody (E2G12) (24). F. To estimate concentration dependent oligomerization, various concentrations of rPfP2 solution in PBS (pH 7.4), ranging from 0.01 ng/ ml to 100 mg/ml were incubated at 4°C for 48 hrs, and 1 ng of rPfP2 from each solution, diluted in SDS-PAGE loading buffer, was resolved on 12% SDS-PAGE with reducing agent (10 mM DTT) followed by immunoblotting and probing with E2G12 (24).
Figure 2. Gel filtration profile of rPfP2, PfP2 mutants and deletion proteins.

A. FPLC profile of 1 mg each of purified recombinant proteins (P2, M1, M2 and M3). Arrow heads show the elution positions of marker proteins, BSA (66 kDa) and Aprotinin (6.5 kDa). Y axis represents UV absorption of protein in arbitrary unit (AU) and X axis shows the fraction number. All the gel filtration runs were performed under reducing condition using buffer containing 10 mM DTT. B. I, II: Gel filtration profiles for rPfP2 and M3 (C12A & C53Y) proteins under non reducing conditions. About 1 mg each of rPfP2 and M3 proteins were injected in the Superdex-75 gel filtration column and fractions were eluted. Bar in B.II. indicates void volume (>300 kDa).

C. I. Coomassie stain of 12% SDS-PAGE of the Peak 1 eluted from A I-IV FPLC runs, showing intact PfP2, M1, M2 and M3 proteins. II. rPfP2 was incubated with agarose dextran cross-linked Superdex-75 beads and subjected to different wash stringencies (2x, 5x and 10x washes with PBS) to detect for weak binding interactions with the chromatography column material. I – input, B – beads, S – post-bead supernatant.

D. I, II: Gel filtration profiles of 1 mg each of recombinant P2del20 and P2del40 proteins, respectively, run under reducing conditions with 10 mM DTT. Insets show Coomassie stained SDS-PAGE profiles of Peaks 1 and 2. Each run was repeated at least three times, and a representative plot is shown.
Figure 3. P2 is largely an alpha helical protein and helicity does not change upon cysteine replacements. Far-UV circular dichroism (CD) spectra of the protein samples were recorded under native condition as also in the presence of reducing agent DTT (5 mM) on a JASCO-J810 spectropolarimeter (Jasco, Hachioji, Japan) at 30 °C, using a 0.1 cm cell and a slit width of 2 nm. 30 µM of each recombinant protein in Phosphate Buffered Saline (PBS, pH 7.4) were subjected for secondary CD. Each spectrum is the average of 3 wavelength scans. Near-UV CD spectra for all constructs were acquired under non-reducing conditions. Each spectrum is the average of 10 wavelength scans acquired using a 1 cm cell and a slit width of 2 nm. Samples were equilibrated for at least 10-12 hrs before CD measurements. A-B Overlay of far-UV CD spectra of P2 and point mutants under non-reducing (A) and reducing (B) conditions. C-D Overlay of far-UV CD spectra of P2 and its deletion constructs under non-reducing (C) and reducing (D) conditions. E-F Overlay of near-UV CD spectra of P2 and its point mutants (E) as well as deletion constructs (F).
Figure 4. Gel filtration profiles of PfP2 from total parasite protein extracts from a synchronized *P. falciparum* culture at 18 and 30 hrs post merozoite invasion. A and C. Gel filtration profiles of about 3 to 4 mg of total parasite protein extracts in PBS from 18 and 30 hrs PMI parasite cultures, respectively, was resolved on a Superdex-200 gel filtration column and fractionated. Arrows indicates the elution positions of marker proteins. B and D. Fractions eluted from A and C were run on 12% SDS-PAGE and immunoblotted using anti-PfP2 monoclonal antibody (E2G12) (24). All fractions were tested, and only those fractions that showed reactivity with anti-PfP2 E2G12 are shown.
Figure 5. Gel filtration profile of PfP2 from infected RBC cytosol (A, B) and RBC ghost (C,D) prepared from a synchronized *P. falciparum* culture at 30 hrs post merozoite invasion. A and C. Gel filtration profiles of 3-4 mg protein from 30 hrs PMI *P. falciparum* infected RBC cytosol and ghost, respectively, using Superdex-200 gel filtration column. Arrows indicate the elution positions of marker proteins. B and D. Fractions eluted from A and C were run on 12% SDS-PAGE and immunoblotted using anti-PfP2 monoclonal antibody (E2G12) (24). All fractions were tested, and only those fractions that showed reactivity with anti-PfP2 E2G12 are shown.
Figure 6. Probing in vitro oligomerization of tetramethyl-rhodamine labeled rPfP2 protein using parasite extracts.

A. Schematic depiction of the time scale of *Plasmodium falciparum* parasite development in erythrocyte. In a 48 hrs life cycle, the parasite changes its morphology from rings, trophozoites to multinucleated schizont stages. All subsequent panels depict reactions where 1 ng of tetramethyl-rhodamine conjugated rPfP2 protein was incubated in 40 µl of various parasite preparations at 37°C for 3 hrs. The mixture was treated with SDS loading dye and resolved on a 12% SDS-PAGE.

B. Labeled rPfP2 was incubated with 4 µg parasite protein extracts from each of 6, 12, 18, 24, 30 and 42 hrs synchronized *P. falciparum* cultures.

C. Labeled rPfP2 was incubated with 25 µl of pooled gel filtration fractions obtained from 30 hrs parasite cultures (Figure 4B).

D. Labeled rPfP2 was incubated with 25 µl of pooled gel filtration fractions obtained from 18 hrs parasite cultures (Figure 4A).

E. Labeled rPfP2 was incubated with 5 µl of individual gel filtration fractions (number 80-84) from 30 hrs parasite culture (Figure 4B).

F. Rhodamine labeled recombinant GST-PfP1 (24) protein was incubated with 25 µl of pooled gel filtration fractions (65-69 and 80-84) obtained from 30 hrs PMI parasite cultures (Figure 4B). Labeled rPfP2 was used as a positive control.

G. About 25 µl of the two pooled gel filtration fractions (65-69 and 80-84) were boiled for 10 minutes. The supernatant was incubated with rhodamine labeled rPfP2. Rhodamine labelled P2 incubated with the respective fractions without boiling was used as a control.
Erythrocytic stage dependent regulation of oligomerization of Plasmodium ribosomal protein P2
Sudipta Das, Sudarsan Rajagopal, Subramanian Sivakami and Shobhona Sharma

J. Biol. Chem. published online October 11, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.384388

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/10/11/M112.384388.DC1