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Research Article
Thermal Stability Properties of A 35-KDa FK506-binding Protein of *Plasmodium knowlesi*

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Abstract

Background and Objective: A 35 kDa of FK506-binding protein of *Plasmodium knowlesi* (Pk-FKBP35) is a member of peptidyl prolyl cis-trans isomerase (PPIase) consisting of an N-terminal catalytic domain (FKBD) and C-terminal tetratricopeptide repeat domain (TPRD). This study aimed to investigate the thermal stability of full-length Pk-FKBP35 and its domains. **Materials and Methods:** Full-length Pk-FKBP35 and its isolated domain (Pk-FKBD and Pk-TPRD) were overexpressed in *Escherichia coli* BL21(DE3) and purified. Thermal stability of purified protein was measured based on the fluorescence signal of 8-anilino-1-naphthalenesulfonic acid (ANS) at the temperature ranging from 25-95°C. **Results:** The thermal denaturation curve for Pk-FKBP35 shows a cooperative transition with a T_m of 56.49±3.05°C, whereas calculated T_m for Pk-FKBD and Pk-TPRD were 60.67±2.81 and 53.64± 4.21°C, respectively. Higher stability of Pk-FKBD might be due to the high content of β-sheet secondary structure. Thermal unfolding profiles of Pk-FKB35 and Pk-FKBD in the presence of PPIase substrate were considerably different as compared to that of in the absence of the substrate, which might be due to structural stabilization by the substrate. **Conclusion:** The thermal stability property of Pk-FKBP35 is characterized by two events of inter-domain destabilization and substrate-mediated stabilization.

Key words: Peptidyl prolyl cis-trans isomerase, FK506-binding protein (FKBP), *Plasmodium knowlesi*, malaria, thermal stability

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plasmodium knowlesi is the 5th malaria causing parasite which is widely distributed in southeast Asia, especially Borneo of Malaysia[1]. In contrast to the other malaria parasites, *P. knowlesi* naturally occurs in long-tailed and pig-tailed macaques and can be transmitted from monkeys to humans by the bite of an infected mosquito[2]. In Malaysia, this parasite is currently known to be the most common cause of malaria and associated with the highest risk of severe disease[3].

It was previously reported that FK506 exhibited antimalarial activity through inhibition of a parasite protein, known as 35-kDa FK506-binding protein (FKBP35), a member of peptidyl prolyl cis-trans isomerase (PPIase)[4-6]. The PPIase is known as a group of enzymes able to catalyze slow isomerization of cis-proline bond during protein folding[7]. The cellular functions of FKBP35 might be related to folding machinery or protein synthesis inside the parasite cells[1,5,6]. The FK506 is known to inhibit catalytic PPIase activity of this protein, which further disrupted its cellular function which then raised lethal effect on the parasite cells[1,6,8,9].

Previous studies on FKBP35 from *P. falciparum* and *P. vivax* demonstrated that FKBP35 is a multi-domain protein consisting of an FK506-binding domain (FKBD) followed by a tetratricopeptide repeat domain (TPRD)[1]. The FKBD plays as the binding site of FK506 and responsible for catalytic PPIase activity with high similarity to human FKBP12 in its amino acid sequence[9,10]. While functional studies of this protein were extensively done in *P. falciparum* and *P. vivax*, there is no report so far for FKBP35 from *P. knowlesi* (Pk-FKBP35). Besides, by far no reports for thermal stability properties of FKBP35 from any Plasmodium parasite, including *P. falciparum*, *P. vivax* or *P. knowlesi*, are available. With regards to Pk-FKBP35, the only report was dealing with the structural dynamic and the three-dimensional model[11] of Pk-FKBP35. This study revealed the structural region responsible for the conformational changes of this protein with no association to its thermal stability.

Study on thermal stability of Pk-FKBP35 is relevant to the idea of targeting this protein in antimalarial drug development[6]. Rathore and Rajan[12] reported that thermal stability of proteins plays important roles in pipeline of drug developments. In particular, understanding thermal stability of target proteins should leads to discover the structural region responsible for the stability. This current study described the thermal stability properties of Pk-FKBP35, which is characterized by two events of inter-domain destabilization and substrate-mediated stabilization.

MATERIALS AND METHODS

Location and time: The experiments were conducted in Biotechnology Research Institute, Universiti Malaysia Sabah, from February-November, 2015.

Materials: All buffer materials and salts were purchased from Nacalai Tesque (Kyoto, Japan). The His-Trap Ni-NTA and the protein molecular weight marker were acquired from GE Healthcare (Singapore). Isopropyl-thio-b-D-galactopyranoside (IPTG) was procured from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21(DE3) cells and kanamycin were bought from Invitrogen (Carlsbad, CA, USA). Fluorescent probe bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt) was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Protein preparation: All proteins were prepared in a His-tagged forms. The expression systems of pET29b-FKBP35, pET29b-FKBD and pET29b-TPRD were used to overproduce full-length Pk-FKBP35, catalytic domain (Pk-FKBD) and TPR-domain (Pk-TPRD), respectively. These expression systems were constructed previously by Carlmond Goh Kah Wun (Universiti Malaysia Sabah). The nucleotide sequences of the genes encoding mutant proteins were verified by sequencing on an ABI Prism 310 DNA Sequencer (Applied Biosystems, California, USA). All these expression systems were transformed into *Escherichia coli* BL21(DE3) for overproduction purposes.

Overproduction and purification of the proteins were carried out as described previously for FKBP35 from *P. falciparum* (Pf-FKBP35)[1]. Production levels of the recombinant proteins in *E. coli* cells and their purities were analyzed by SDS-PAGE using 15% polyacrylamide gel[13], followed by staining with Coomassie Brilliant Blue R250. Protein concentrations were determined by using UV absorption on the basis that the absorbance at 280 nm of a 0.1% (1 mg mL^{-1}) solution is 0.73, 0.65 and 0.76 for full-length Pk-FKBP35, Pk-FKBD and Pk-TPRD, respectively. These values were calculated by using[14] $\varepsilon = 1576\ M^{-1}\ cm^{-1}$ for Tyr and 5225 $M^{-1}\ cm^{-1}$ for Trp at 280 nm.

Fluorescence measurements: Fluorescence measurements were conducted essentially based on previous methods[15,16]. The use of ANS was based on the fact that ANS is widely used in the thermal stability analysis of protein and considered as high sensitivity of fluorescent dyes[17]. Briefly, a 1 µM of protein in 20 mM of Tris-HCl buffer with pH 8.0 was mixed and

allowed to equilibrate with 10 μM 8-anilino-1-naphthalene sulfonic acid (ANS). Prior to the use, ANS stock solution was made in methanol and stored at 4°C, in aluminum foil covered vials and used within 1 week of preparation. The samples prepared are then heated with different temperatures from 25-95°C for 15 min. Fluorescence and absorption measurements were carried out on Hitachi F-4010 fluorescence spectrophotometer and Hitachi U-3010 UV-visible spectrophotometer. All the fluorescence spectra were corrected by using the software provided in the instrument. Fluorescence readings were taken for each sample in quartz cuvette in at 370 nm and collected emission spectra from 400-600 nm. The thermal unfolding was derived as a function of fraction folded of protein and temperature. Fraction folded was calculated based on the maximum ANS fluorescence intensity at each temperature. The melting temperature (T_m) was determined by obtaining the first derivative to the curve and identifying the curve's maximal point. To determine the effect of substrate on the thermal stability, the analysis was also performed in the presence of 25 uM (ALPF) Suc-Ala-Leu-Pro-Phe-pNa substrate, a substrate for PPIase activity. This concentration selected under consideration of the concentration used for activity assay.

Statistical analysis: Data were expressed as mean±standard deviation of at least three independent experiments and summarized using descriptive statistics.

RESULTS

Protein preparation: The primary structure of all proteins was shown in Fig. 1, in which all proteins were expressed as 6xHis-tagged forms at their N-terminal. The secondary structure of full-length Pk-FKBP35 was also displayed in Fig. 2 showing that the full-length Pk-FKBP35 is predicted to be dominated by helical secondary structures. All proteins were successfully expressed in *E. coli* BL21 (DE3) in soluble forms and purified under Ni-NTA chromatography followed by size exclusion chromatography (Fig. 3). The size of full-length Pk-FKBP35 is about 37 kDa in SDS-Page, while FKBD and TPRD have an apparent size of 16 and 21 kDa, respectively.

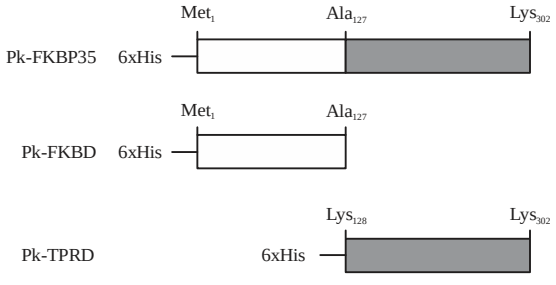

Fig. 1: Primary structure of full-length Pk-FKBD and its domains
The proteins were expressed in 6X-His tagged forms at their N-terminals

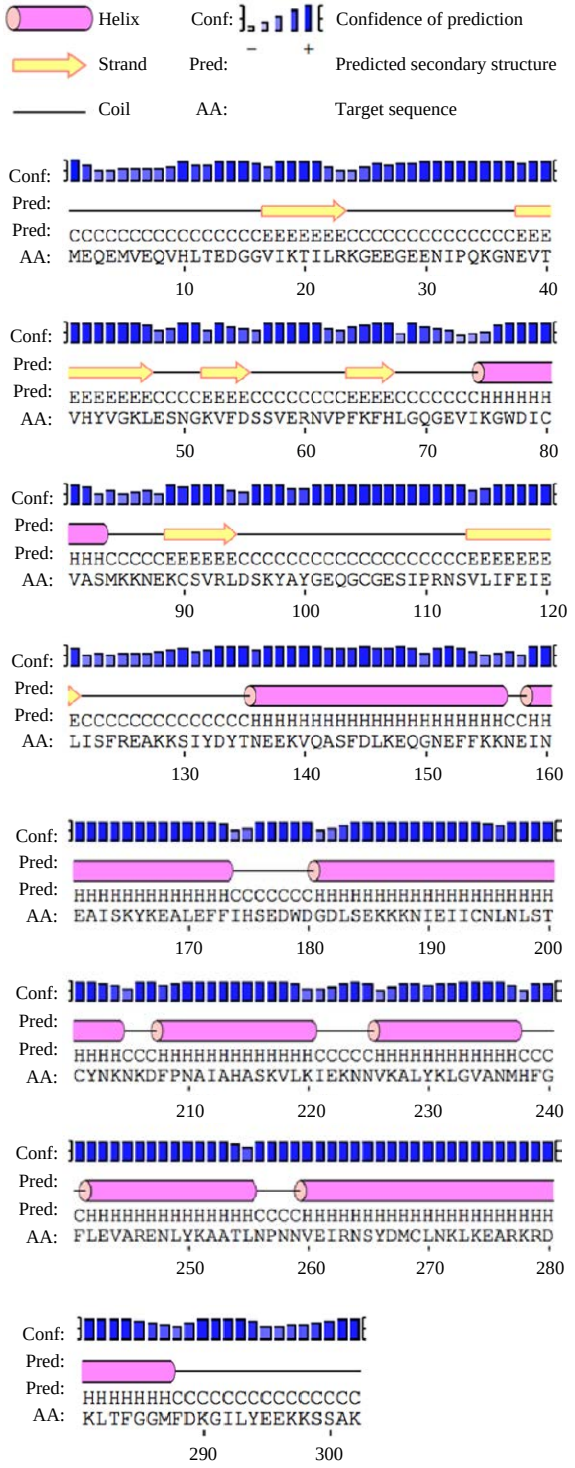

Fig. 2: Secondary structure of full-length Pk-FKP35 as predicted using PSIPRED server

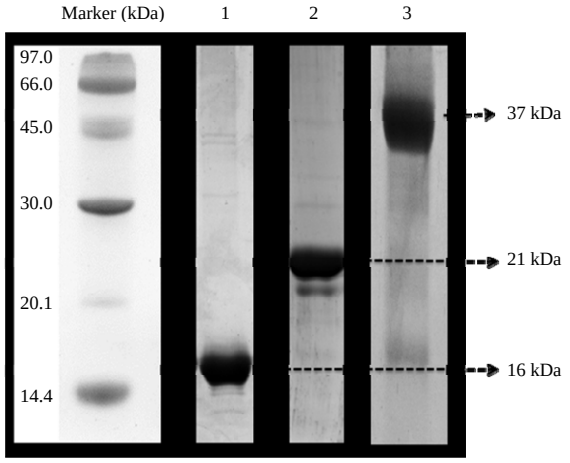

Fig. 3: 15% SDS-page of purified full-length Pk-FKBP35, Pk-FKBD and Pk-TPRD

Marker lane refers to the mixture of proteins with the apparent sizes (kDa) displayed beside the lane as the reference to determine the apparent size of full-length Pk-FKBP35 and its domains. Lane 1, 2 and 3 refer to Pk-FKBD, Pk-TPRD and full-length Pk-FKBP35. The apparent size (kDa) of full-length Pk-FKBP35 and its domains are indicated by the arrows

Table 1: Melting temperature (T_m) of Pk-FKBP35 and its derivatives

Protein	T_m (°C)
Pk-FKBP35	56.49±3.05
Pk-FKBD	60.67±2.81
Pk-TPRD	53.64±4.21

ANS-fluorescence spectroscopy: The results in Fig. 4 showed the ANS fluorescence intensity changes in the presence of Pk-FKBP35 or its derivatives (FKBD or TPRD) at various temperatures. The fluorescence intensity was found to be almost stable at the temperature higher than 80°C suggesting that the proteins were completely unfolded. While slight increase was observed in this region, however, it is not significant and thus considered to be a steady state. Meanwhile, the fraction below 30°C was suggested to be in a folded state as there are no significant changes in ANS intensity at this region. Melting temperatures (T_m) of the proteins were shown in Table 1, which were calculated from their thermal denaturation curve (Fig. 4). Table 1 showed that FKBD exhibited higher T_m (60.67±2.81°C) than full-length Pk-FKBP35 (56.49±3.05°C) and TPRD (53.64±4.21°C).

PPIase substrate effects on ANS-fluorescence spectroscopy: Figure 5 showed ANS fluorescence intensity changes, in the presence of Pk-FKBP35 or FKBD, with PPIase substrate at various temperatures. The ANS fluorescence intensity of Pk-FKBP35 and FKBD in the presence of PPIase substrate were found to increase at the higher temperature, even at the

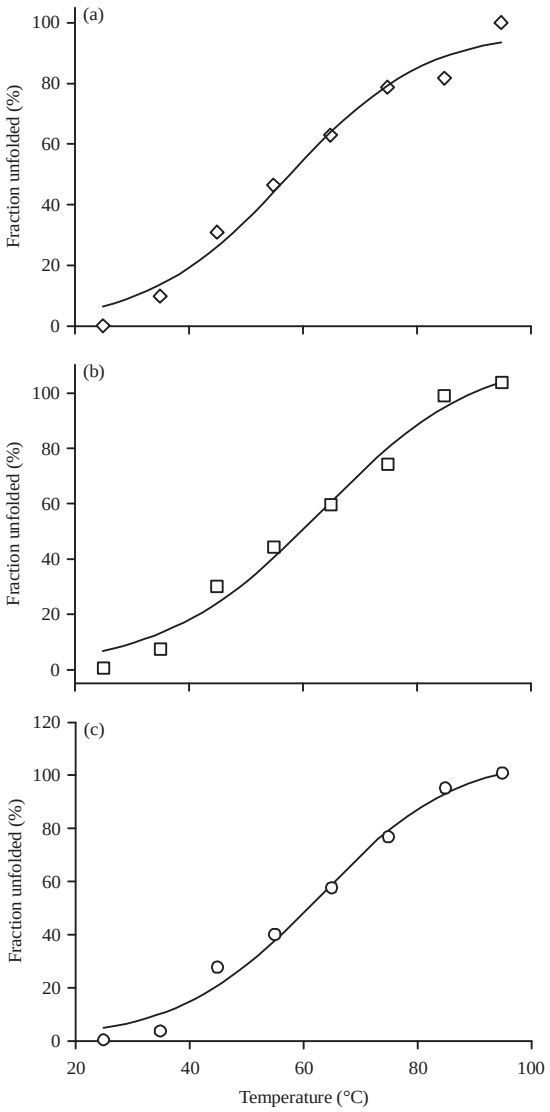

Fig. 4(a-c): Thermal denaturing curve of full-length Pk-FKBP35, Pk-FKBD and Pk-TPRD in the absence of Suc-ALPF-pNA substrate

The curve was derived from maximum fluorescence intensity of ANS at various temperatures

temperature higher than 80°C. Nevertheless, the denaturation curves derived from the increasing of ANS fluorescence signal in the presence of substrate displayed the presence of a transition phase. The T_m, thus, was not feasible to be calculated because did not fit with the curve equation.

DISCUSSION

According to the current study the melting temperature (T_m) of full-length Pk-FKBP35 and TPRD were similar, whereas, T_m of FKBD was slightly higher than that of full-length

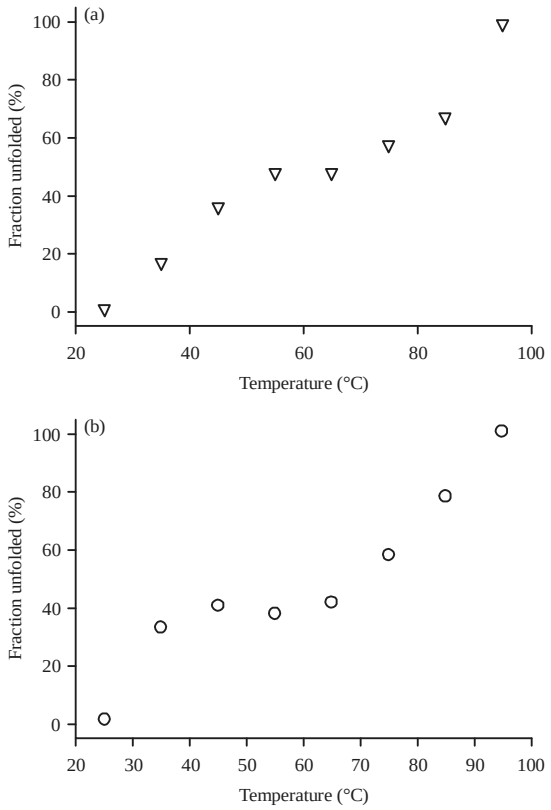

Fig. 5(a-b): Thermal denaturing curve of full-length Pk-FKBP35 and Pk-FKBD in the presence of Suc-ALPF-pNA substrate

Curve was derived from maximum fluorescence intensity of ANS at various temperature in the presence of Suc-ALPF-pNA substrate

Pk-FKBP35 and TPRD. High stability of FKBD might be related to the high content of β-sheet structure in this domain. Indeed, the secondary structure prediction of this protein demonstrated that FKBD is dominated by β-sheet structure. This was also in good agreement with structural modeling of FKBP35 demonstrating that FKBD is rich in β-sheet structure, while TPRD has no β-sheet structure[11]. To note, FKBD displays high similarity to human FKBP12, which is structurally dominated by β-sheet structure[18]. Meanwhile, the TPRD motif was reported to be fully composed helical structure that known to be less stable as compared to β-sheet structure[9,19]. The higher thermal stability of β-sheet structure as compared to helical structure was previously observed in various proteins[20-22]. Besides, stability of FKBD was also supported by previous in silico analysis demonstrating that this domain is more rigid as compared to TPRD[11]. It was known that overall flexibility is reduced when thermostability is increased[23], thus it is acceptable to have FKBD to be thermally more stable than TPRD as this domain is found to be more rigid[11]. Considering that FKBD is a catalytic domain, this result is interesting since the catalytic domain is known to be relatively less stable allowing this domain to capture the substrate in high freedom. Yet, this does not reflect the flexibility of the substrate-binding pocket of this protein. There is a possibility that substrate binding pocket remains flexible allowing the catalysis to occur efficiently.

It was also observed that full-length Pk-FKBP35 exhibited lower T_m as compared to Pk-FKBD. The T_m of full-length Pk-FKBP35 is supposedly an average of T_m of the domains (FKBD and TPRD). To note, structurally, full-length FKBP35 is a combination of FKBD and TPRD, which leads to an assumption that T_m of full-length Pk-FKBP35 is supposedly higher than T_m of both domains. This assumption is due to the general phenomena on protein stability as results of the cumulative effect of local interactions between side chains, the polypeptide backbone, domains and subunits[24-26]. Nevertheless, lower T_m of full-length Pk-FKBP35 as compared to FKBD suggested that this protein did not follow the cumulative effect on its stability. Rather, structural destabilization is believed to be the factor causing this issue. The destabilization is believed to occur mostly in the interface of FKBD and TPRD to prevent steric clash among atoms in these domains. The destabilization at the interface also allows the domains to have structural freedom to move in related to their functions. This is proposed as an "Inter-domain destabilization" event, a phenomenon that was also observed in other multi-domain proteins[27,28]. In addition, according to Bhaskara and Srinivasan[29], the isolated domains of multi-domain proteins were often found to be significantly less stable than that of in full-length polypeptide chains. Similarly, Batey *et al.*[30] also reported the possibility of the native state of domains is stabilized by the interaction in the fully folded protein, which leads to be more stable than in their isolated forms. This leads to the possibility of targeting inter-domain of FKBP35 for antimalarial drug design through destabilization of this protein. A similar strategy was also previously proposed by Bexter *et al.*[31] for the development of diabetes drug targeting interdomain of MitoNEET protein.

It was noticed that the ANS intensity of the proteins in the presence of the substrate kept increasing at the temperature higher than 80°C, which remarkably differences to that of in the absence o the substrate. The reason behind the increase of the ANS intensity at the temperature higher than 80°C remains unclear, however, this suggested that the substrate might have some roles in thermal-stability of full-length

Pk-FKBP35 and FKBD. It is widely known that binding to the substrate was usually accompanied by stabilization of the substrate-binding region[32-35]. Even though the current result is unable to determine quantitative effect of the substrate on thermal stability of Pk-FKBP35, it is clear that the curve was significantly different to that of in the absence of substrate. It is interesting to note that the complex structure of FKBD of *P. falciparum* with FK506, a PPIase inhibitor, showed significant structural changes on this protein mainly on β-sheet structures[8,10]. Meanwhile, the complex structure of Suc-Ala-Leu-Pro-Phe-pNA and FKBD of *P. vivax* showed subtle changes in the ligand-flanking loops, namely the β3-β4 loop, β4-α1 loop and β5-β6 loop[36]. Whether or not these subtle changes associated with the thermal stability of this protein remain to be confirmed. Nevertheless, this is the first result on the effect of substrate on thermal stability of Pk-FKBP35 that indicates significant changes in the stability upon the addition of the substrate. This also leads to a promising strategy of development of drug mimicking the substrate of FKBP35 that would further lead to lose partial of even total function of each domain. Indeed, high throughput screenings of small molecules destabilizing diseases causing proteins were widely done in drug discovery[37,38].

CONCLUSION

The T_m value of Pk-FKBD was shown to be higher than that of Pk-TPRD which might be due to differences in β-sheet structure composition. The result also showed that T_m of full-length Pk-FKBP35 is lower than Pk-FKBD, yet higher than Pk-TPRD suggesting an inter-domain destabilization event in this protein. Besides, PPIase substrate was also found to have some effects on the thermal stability of the protein. In overall, this finding is useful in providing a platform for the development of novel antimalarial drug targeting destabilization of this protein.

SIGNIFICANCE STATEMENT

This study discovers thermal stability properties of Pk-FKBP35, a protein involved in infection of *Plasmodium knowlesi* that can be beneficial for development of antimalarial drug with no resistance effect. This study will help the researcher to uncover the critical areas of structural regulation on thermal stability of FKBP35 that many researchers were not able to explore. Thus, a new theory on inter-domain destabilization and ligand-mediated stabilization may be arrived at.

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