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Research paper

Structural and functional characterization of an iron–sulfur cluster assembly scaffold protein-SufA from *Plasmodium vivax*

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ABSTRACT

Iron–sulfur (Fe–S) clusters are utilized as prosthetic groups in all living organisms for diverse range of cellular processes including electron transport in respiration and photosynthesis, sensing of ambient conditions, regulation of gene expression and catalysis. In *Plasmodium*, two Fe–S cluster biogenesis pathways are reported, of which the Suf pathway in the apicoplast has been shown essential for the erythrocytic stages of the parasite. While the initial components of this pathway detailing the sulfur mobilization have been elucidated, the components required for the assembly and transfer of Fe–S clusters are not reported from the parasite. In *Escherichia coli*, SufB acts as a scaffold protein and SufA traffics the assembled Fe–S cluster from SufB to target apo-proteins. However, in *Plasmodium*, the homologs of these proteins are yet to be characterized for their function. Here, we report a putative SufA protein from *Plasmodium vivax* with signature motifs of A-type scaffold proteins, which is evolutionarily conserved. The presence of the $[Fe_4S_4]^{3+}$ cluster under reduced conditions was confirmed by UV–visible and EPR spectroscopy and the interaction of these clusters with the conserved cysteine residues of chains A and B of *Pv*SufA, validates its existence as a dimer, similar to that in *E. coli*. The H-bond interactions at the *Pv*SufA–SufB interface demonstrate SufA as a scaffold protein in conjunction with SufB for the pre-assembly of Fe–S clusters and their transfer to the target proteins. Co-localization of the protein to the apicoplast further provides an experimental evidence of a functional scaffold protein SufA for the biogenesis of Fe–S clusters in apicoplast of *Plasmodium*.

1. Introduction

Apicoplast, a non-photosynthetic plastid in the apicomplexans has gained a lot of attention in the last two decades as a putative drug target due to its prokaryotic nature and indispensability for the parasite survival (Ralph et al., 2001). It harbors four major metabolic pathways of importance, amongst which the iron sulfur [Fe–S] cluster biogenesis pathway is essential for apicoplast maintenance in the erythrocytic stage of *Plasmodium* (Gisselberg et al., 2013; Haussig et al., 2014). Many of the enzymes involved in major metabolic pathways like IspG and IspH (isoprenoids biosynthesis pathway), lipoate synthase (LipA) and the t-RNA modification enzyme (MiaB) require 4Fe–4S clusters for their activity (Pierrel et al., 2002; Cicchillo et al., 2004; Zepeck et al., 2005; Rekittke et al., 2008; Lee et al., 2010). The functionality of these proteins is further believed to depend on the 2Fe–2S electron transfer protein ferredoxin (Fd) (Rohrich et al., 2005; Kimata-Ariga et al., 2007), which further details the importance of Fe–S clusters and the pathway.

Fe–S cluster biogenesis pathways are conserved in nature, with three distinct pathways reported in the prokaryotes, namely: Isc (iron–sulfur cluster) and Nif (nitrogen fixation machinery) for housekeeping cluster assembly and Suf (sulfur mobilization) to be used under stress conditions. In *Plasmodium*, the Isc and Suf pathways have been shown to exist, where some of the constituent proteins involved in these pathways are targeted to the parasite's mitochondria and apicoplast respectively (Gisselberg et al., 2013; Haussig et al., 2014). All the key molecules participating in the above pathway are nuclear encoded except the SufB which is encoded by the apicoplast genome and interacts with SufC, an ATPase lying in close proximity to the apicoplast membrane (Kumar et al., 2011). Another component of the Suf system, SufS is active in the apicoplast and along with SufE transfers the sulfur from cysteine to SufBCD complex (Gisselberg et al., 2013; Charan et al., 2014). While the mobilization of sulfur catalyzed by

Abbreviations: Fe–S, iron–sulfur; Suf, sulfur mobilization; *Pv*, *Plasmodium vivax*; EPR, electro paramagnetic resonance; Isc, iron–sulfur cluster; Nif, nitrogen fixation.

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SufSE complex have been extensively studied, the subsequent steps detailing the assembly of mobilized sulfur and iron to form Fe–S clusters and their transfer to the apo-proteins is still functionally uncharacterized in *Plasmodium*. Thus, to further unfold the importance of this pathway in the parasite, we have characterized one of the components of the Fe–S cluster biogenesis pathway; an iron sulfur cluster assembly accessory protein (SufA, putative) from *Plasmodium vivax*. We report apicoplast specific localization of the full length recombinant *Pv*SufA protein and in vitro formation of [4Fe–4S] clusters on this protein. In addition to this, we also analysed the interaction of *Pv*SufA with other components of the pathway like *Pv*SufB and *Pv*SufC in silico to explore the interacting partners involved in the assembly and transfer of the [4Fe–4S] clusters to the apo-proteins.

2. Materials and methods

2.1. Patient sample collection, RNA isolation and cDNA preparation

Clinically proven (using conventional microscopy and rapid diagnostic tests) *P. vivax* infected patient venous blood samples were collected in acid citrate dextrose (ACD) from patients admitted to S.P. Medical College and associated group of hospitals, Bikaner, India. A formal approval of participating Institute's Human Ethics Committee (approval no. IHEC-35/13-14) and patient's consent was taken prior to collection for further studies. Blood was immediately subjected to density gradient centrifugation (Histopaque 1077, Sigma Aldrich, USA) to separate the infected RBCs which were further stored in Tri Reagent (Sigma Aldrich, USA). Total RNA and DNA were isolated from these samples using the manufacturer's protocol. The presence of *P. vivax* mono infection was confirmed by diagnostic PCR based on 18S rRNA gene (Das et al., 1995; Kochar et al., 2005) and 28S rRNA gene (Pakalapati et al., 2013). RNA from confirmed *P. vivax* cases was used to prepare total cDNA using QuantiTect Reverse Transcription Kit (QIAGEN, Germany).

2.2. Multiple sequence and phylogenetic analysis

To understand the evolutionary position of Indian *Pv*SufA protein, a phylogenetic tree was constructed using MEGA 6.0 (Tamura et al., 2006). Multiple sequence alignment was done for Indian *Pv*SufA (gene ID: KU556730) with the SufA sequences available from different apicomplexans and prokaryotes at NCBI using CLUSTAL Omega (Sievers et al., 2011). The evolutionary history was inferred using the maximum-likelihood method (Jones et al., 1992). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985) where the analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 79 positions in the final dataset. To obtain the phylogenetic tree, neighbor-join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using a JTT model, and then the topology with superior log likelihood value was selected (Tamura et al., 2006).

2.3. Cloning and purification of recombinant protein

The full length coding *Pv*SufA gene (PlasmoDB gene ID: PVX_080115) was amplified from total cDNA of *P. vivax* using forward 5′ GCGGGATCCATGGCAACGACAAAGGC3′ and reverse primer 5′ GCGCCATGGCTAAACATTGAATGACTTCCC 3′. The amplicons were cloned in pRSETA expression vector (Invitrogen, USA), and both the amplicons and the clone were confirmed using T7 and gene specific primers. The obtained sequences were analyzed for conserved residues and motifs. Targeting to the apicoplast and presence of signal or transit leader sequence for apicoplast was confirmed using PlasmoAP (Foth et al., 2003). The recombinant protein was induced with 0.5 mM IPTG (MERCK, India) for 6 h at 37 °C in *Escherichia coli* Rosetta BL21(DE3) pLysS cells (Promega, USA) and the expression was checked on a 15% SDS PAGE. Recombinant (6XHis)-*Pv*SufA (~21 kDa) was purified through a Ni-NTA matrix (QIAGEN, Germany) and presence of recombinant protein was confirmed by western blotting using anti-His antibodies (QIAGEN, Germany).

2.4. Antibody raising and immuno-localization

Purified *Pv*SufA protein (30 μg) was formulated with Freund's adjuvant (Merck, India) and injected in 4–5 weeks old female Swiss Albino out-bred mice followed by three booster doses at regular intervals of 21 days (Edward and David, 1988). All the protocols were approved by Institute's Animal Ethics Committee (approval no. IAEC/RES/18/30). The specificity of the *Pv*SufA protein antibodies in the serum samples was confirmed by western blot analysis and antibody titre of each sera sample was checked using ELISA. The confirmed sera samples with good antibody titre were further used for immuno-fluorescence microscopy. For co-localization of the *Pv*SufA protein, *P. vivax* parasite infected blood smears (from patients) were fixed with cold methanol, permeabilized with 0.05% Saponin (30 min) and 0.1% Triton X-100 (4 min) followed by overnight blocking in 3% Bovine Serum Albumin (BSA) at room temperature. Cells were further incubated with *Pv*SufA antiserum (1:1000 dilution for 4 h) and goat anti-mouse IgG FITC conjugate (1:2000 dilution for 1 h and 30 min; MERCK, India) at 25 °C. Counterstaining of the parasite nucleus was done using DAPI (Life Technologies, USA) for 10°min and apicoplast membrane using Qdot® 585 Streptavidin conjugate (Life Technologies, USA) for 1 h at room temperature (Jelenska et al., 2001). Intermittent washing with PBS was performed in between each step. The cells were finally mounted with VECTASHIELD (Vector Laboratories, USA) and viewed in a confocal laser scanning microscope (Leica TCS SP5) under a 63X oil immersion lens.

2.5. Biochemical and EPR spectra analysis of PvSufA protein

Purified *Pv*SufA (4 μM) was incubated anaerobically at 25 °C with 5-fold molar excess of both Na_2S and $Fe(NH_4)_2(SO_4)_2$ for 3 h in the presence of 5 mM dithiothreitol prepared in 0.1 M Tris–HCl (pH 8.0), to reconstitute the Fe–S clusters on the protein. The unbound iron and sulfur were removed using Amicon Ultra-4 filters (Merck, Germany). The amount of iron and sulfur bound was determined using Ferrozine (Fish, 1988) and methylene blue (Siegel, 1965) colorimetric assays respectively.

Further the above reconstituted protein complexed with Fe–S clusters was analyzed by EPR spectroscopy to check for the oxidation state of bound Fe–S cluster (Zeng et al., 2007). Briefly, the samples were diluted to a concentration of 5 μM in 20 mM phosphate buffer containing 0.5 M NaCl (pH 7.4) followed by incubation with 5 mM ammonium persulfate for 30 min to oxidize *Pv*SufA for spectra analysis. X-band EPR spectra were recorded at 100 K on a JES-FA200 spectrometer. Parameters for recording the EPR spectra were typically 15–30 mT/min sweep rate, 0.63 mT modulation amplitude, 9.44 GHz frequency, and 4 mW incident microwave power with a sweep time of 2 min.

2.6. Molecular modeling

Crystal structure of *E. coli* SufA (PDB ID: 2D2A) with resolution of 2.7 Å was used as a template for *Pv*SufA protein structure prediction based on the results obtained from HHpred online server (Söding et al., 2005). After removing the signal and transit peptide sequence from the *Pv*SufA protein sequence, a three dimensional model was built using the program MODELLER 9v11 (Sali and Blundell, 1993; Eswar et al., 2006). The best model with minimum DOPE score was selected and further evaluated for quality and atomic content using different online servers like PROCHECK (Laskowski et al., 1993), VERIFY3D (Eisenberg et al., 1997), ERRAT (Colovos and Yeates, 1993), WHAT IF