Charged gatekeeper residues alone can't determine the nucleotide specificity of succinyl-CoA synthetase (SCS) of P. falciparum

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Abstract

Understanding the molecular basis of substrate specificity of an enzyme is very crucial to its functional importance in any biological system. Previously, we had experimentally validated a novel phenomenon using an enzyme engineering approach, to alter the substrate specificity via modification of the electrostatic properties of the gatekeeper residues in succinyl-CoA synthetase (SCS) of Blastocystis. The multiple sequence alignment (MSA) of SCSβ subunits from phylogenetically diverse organisms, depicted P. falciparum SCS to club with ADP-forming human, bovine and murine SCS enzymes. In the present study, we have explored the gatekeeper residues of P. falciparum SCS (PfSCS), an enzyme crucial for the generation of a metabolic intermediate- succinyl-CoA, required during the mosquito stages of the Plasmodium. With the construction of various gatekeeper mutants, structural modeling and enzyme kinetics experiments, our study concluded that PfSCS is an ADP-forming enzyme (KmATP=48 μM). Introduction of the exclusively charged- positive (Lys & Lys) & negative gatekeeper residues (Glu & Asp), demonstrated significant reductions in the ATP affinity, while no significant GDP-forming potential was recorded. Interestingly, simultaneous refolding of the nucleotide-binding site containing PfSCSβ subunit with the Blastocystis SCSα produced the active enzyme conformation. Therefore, the present study concluded that only electrostatic interactions at the gatekeeper region are not sufficient enough to alter the substrate specificity in PfSCS, as in case of Blastocystis SCS and further structural analysis is warranted with particular focus on the binding site architecture of PfSCS.

Introduction

In any biological system, the substrate specificity is a characteristic property of the enzymes. There are two landmark models to describe substrate specificity of an enzyme- 'lock and key model' [1] proposing a rigid fit; while the 'induced fit model' [2] suggested a flexible nature of the enzyme to fit the substrate. At the molecular level, the substrate specificity is best described by the molecular interactions of a protein and its substrates. The free energies of the hydrogen bonds between a protein-substrate and the propensity of certain amino acids around the substrate, play a critical role in the substrate specificity of an enzyme [3]. In addition, other weak interactions such as Van der Waals and electrostatic interactions [4], between the protein and its substrate, also have significant contribution in the substrate specificity of an enzyme; especially, when the proteins have to discriminate between two similar substrates eg. adenine and guanine (A & G) in nucleotide-binding proteins. Basu et al. reported that a strong ligand-free electrostatic potential (ESP) could discriminate between A/G binding sites and hence established the role of an electrostatic component in the molecular discrimination of A & G. Previously, the electrostatic potential arising from the charged amino acids inside the active site of subtilisin enzyme, had been shown to be functionally significant [5]. However, the role of other charged amino acids near or outside the active site has not been investigated thoroughly. In 2008, Hamblin et al. proposed an electrostatic gatekeeper effect, in which the nucleotide access is controlled by the charged amino acids (gatekeeper residues) outside the binding site of the succinyl-CoA synthetase (SCS) of *Blastocystis* - a human intestinal parasite [6]. Recently, we have experimentally

demonstrated the 'electrostatic gatekeeper effect', where the gatekeeper residues were found to be critical for nucleotide specificity in *Blastocystis*SCS [7]. Interestingly, this study also established a novel enzyme engineering approach, where the switching of the charge of the gatekeeper residues from positive to negative, demonstrated that the ADP-forming SCS could also utilize GTP. Surprisingly, two binding site modifications in addition to the charge switching, resulted in a complete reversal of an ADP-forming SCS to GDP-forming SCS.

To further signify the role of gatekeeper residues in determining the nucleotide specificity, we explored another model enzyme- SCS of*P. falciparum* . *P. falciparum* is an important human parasite causing a significant infectious disease- malaria, with ˜219 million clinical cases and ˜0.43 million deaths worldwide (WHO malaria report, 2018). The first line of defense for *P. falciparum* malaria are artemisinin combination therapies (ACTs). However, the emergence of resistance against ACTs is a matter of great concern, as was with the previous generation of antimalarials such as chloroquine, sulphadoxine and pyrimethamine. Therefore, a great amount of efforts are currently being devoted to identify of novel drug targets for malaria, simultaneously expanding the fundamental understanding of the molecular biology of *Plasmodium* . SCS is a crucial enzyme of the tricarboxylic acid (TCA) cycle, for its unique capability of generating ATP by substrate-level phosphorylation. In *P. falciparum* , however, the TCA cycle has been suggested to be of limited importance [8], yet the parasite synthesize all the TCA cycle enzymes [9]. During the asexual growth of the malaria parasite, the absence of any specific phenotypes in $\Delta K\Delta H/\Delta\Sigma\tilde{}\Sigma$ &$\Delta\Sigma\tilde{}\Sigma/\Delta\Sigma\Delta H$ KOs (knock out lines) [KDH- α-ketoglutarate dehydrogenase, SCS- succinyl-CoA synthetase α subunit and SDH-*SDH* flavoprotein subunit] indicated a metabolic plasticity in the TCA cycle [10]. Unlike the asexual stages of *P. falciparum* , the SCS is significant in terms of maintaining the reserves of succinyl-CoA, as an initial substrate for heme biosynthesis along with glycine for its sexual stages [11]. The present study explored the alteration of the charge of the gatekeeper residues and its subsequent effect on the substrate specificity of *Pf* SCS.

Material and methods

Computational analysis of the SCS subunits

SCS is composed of two subunits- SCSα & SCSβ, whereas the SCSβ subunit carries the only nucleotide-binding site. The amino acid sequences of the SCSβ subunits from phylogenetically diverse organisms were retrieved from UniProtKB and respective details are summarized in **Table 1** . A multiple sequence alignment (MSA) of these sequences was performed using ClustalO. The alignment output representation was performed by Boxshade server. Weblogos were also generated from the respective alignments of the ADP-forming and GDP-forming SCS to identify the most frequently present gatekeeper residues. After identification of the gatekeeper residues from the MSA, various mutants were designed in an attempt to alter the charge of the gatekeeper residues, details are summarized in **Table 2** . Structure models were generated for the wild-type and various mutant *Pf* SCSβ subunits by using Modeler 9v13, with the following templates- *E. coli* SCS (PDB-1CQI) [12] and pig SCS (PDB-2FP4) [13]. The models were further analyzed by Ramachandran scatter plots and DOPE-scores. The electrostatic surfaces of the gatekeeper regions were also constructed using eF-surf server and visualized using PDBjViewer [14].

Determination of the nucleotide specificity of native*Pf*SCS enzyme

The nucleotide specificity of the native *Pf* SCS enzyme was determined from the lysate of the cultured *P. falciparum* strain 3D7, as described by [15]. Briefly, the parasites were grown in human erythrocytes using 2% hematocrit in RPMI-1640 supplemented with 10% human serum. The lysate was prepared by saponin lysis and ultra-sonication of the cultured parasites, centrifuged at 14500 RPM for 15 minutes at 4 ºC. The supernatant was collected and enzyme assays were performed as described in [6]. Briefly, the assay buffer [129 µM CoA, 10 mM sodium succinate, 50 mM KCl, 10 mM MgCl2, and 50 mM Tris-HCl (pH 7.4)] was used to determine the nucleotide (ATP & GTP) specificity, which was recorded by the formation of a thioester bond in succinyl-CoA at 232 nm.

Cloning, recombinant protein expression and refolding of*Pf*SCS

The *Pf* SCSβ wild-type subunit was amplified using the primer sequences given in **Table 2** . The amplified *Pf* SCSβ gene was ligated in expression vector pET28a vector (Novagen) with 6X His-tag, using the appropriate restriction sites and transformed into*E. coli* (DH5α cells). For recombinant protein expression, the PfSCSβ+pET28a construct was transformed into *E. coli*BL21-CodonPlus® competent cells. The *Pf* SCSβ gatekeeper mutants were generated by a commercially available Q5 site-directed mutagenesis kit (New England Biolabs) and confirmed by sequencing of the constructs for desired mutations at respective positions. The respective primer sequences for substituting the codons are mentioned in **Table 2** . Despite multiple efforts, it was not possible to clone the *Pf* SCSα subunit, hence, the *Blastocystis* SCSα subunit (having >60% identity with *Pf* SCSα) was chosen to generate the refolded *Pf* SCS enzyme.

The protein expression was carried out using standard protocols, optimized in the laboratory (Vashisht et al. 2017). Briefly, the overexpression of the cloned *Pf* SCSβ subunit was induced by the addition of 1mM Isopropyl β-D-1 thiogalactopyranoside (IPTG) after the OD values reached 0.4 - 0.6, and grown for 4 hrs, post-induction. The bacterial cell pellets were reconstituted in lysis buffer [50 mM NaH_2PO_4, 10 mM Tris, 500 mM NaCl, 10 mM imidazole- pH- 8.0] and sonicated. Centrifugation at 14500 RPM for 30 min. at 4 ^{o}C yielded the supernatant and cell debris pellet. The pellet was further processed for isolation of inclusion bodies (IBs) containing the 6X-His-tagged *Pf* SCSβ subunits, washed twice with 1M urea and 1% triton-X100, and finally with 1M urea alone. The IBs were solubilized in solubilization buffer containing [6M Gn-HCL (Guanidine hydrochloride), 10 mM Tris-HCl- pH- 8.0] overnight. The purification of *Pf* SCSβ was carried out by a custom-packed column with Ni-NTA resin (Nucleopore, Genetix Biotech Asia) using a fast-process liquid chromatography system- AKTA Prime, FPLC (GE life sciences). The elutions were collected from the 200 mM imidazole fractions and analyzed by SDS-PAGE. The *Pf* SCSβ subunit was confirmed by western blotting using a commercially available mouse monoclonal antibody raised against 6X-His-tag (Sigma-Aldrich). As mentioned previously, the*Blastocystis* SCSα subunit was used at the time of refolding with*Pf* SCSβ subunit (Vashisht et al. 2017). Both the subunits were again denatured in the solubilization buffer and concentrated using 10 kDa cut-off Centricons (Vivaspin). Optimized refolding was performed in buffer [50 mM Tris-HCl, 25% glycerol, 25 mM DTT & 100 µM $MgCl_2$- pH- 7.2] with rapid dilution (100 fold) of the respective subunits in 1:1 ratio and incubated overnight at 4 ^{o}C. The refolded *Pf* SCS enzymes were again concentrated with a 10 kDa cut-off Amicon stirred-cell (Millipore) for further enzymatic assays.

Enzyme kinetics of the *Pf*SCS (wild-type and various gatekeeper mutants)

Enzymatic assays were performed with optimized conditions in buffer [10 mM sodium succinate, 50 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl- pH- 7.4]. 129 µM coenzyme A (CoA) and ˜30 nanomoles of refolded *Pf* SCS enzymes (wild-type and various gatekeeper mutants) were added in each reaction mix. Varying concentrations of ATP & GTP were used to carry out the enzymatic reaction. The product formation was followed for 10 minutes with 1 minute intervals. A UV-absorbance at 232 nm was recorded in the quartz cuvette of 10 mm path-length corresponding to the formation of a thioester bond in succinyl-CoA. The enzyme kinetics results were analyzed to calculate the Michaelis-Menton constant (K_m) by using Graphpad prism 5.0 software.

Results

Σεχυενχε ανδ μολεχυλαρ μοδελινγ αναλψσις οφ τηε αριους Σ¨Σβ συβυνιτς

The MSA of SCSβ subunit sequences from various organisms is presented in**Fig. 1a** and the respective gatekeeper residues are shaded. Among the ADP-forming SCSβ subunits, the gatekeeper residues are listed in **Table 1.** Human intestinal parasite *Blastocystis* SCS has Lys & Lys (positively charged) gatekeeper residues, while*Pf* SCS has Asp and Tyr (negatively charged and hydrophobic) gatekeeper residues. Another apicomplexan parasite *Toxoplasmagondii* , too has the negatively charged and hydrophobic gatekeeper residues (Asp and Phe), but *Leishmania major* has positive and non-polar (Lys and Gly) gatekeeper residues. Two representative plant species- *Arabidopsis* and *Oryza* have negatively charged and polar/uncharged gatekeeper residues (Glu and Ser, respectively). The ADP-forming SCSβ subunits of *Homo sapiens* , *Bos taurus* ,*Mus musculus* and *Sus scorfa* has the similar gatekeeper residues as of *P. falciparum* (Asp and Tyr), how-

ever, the GDP-forming SCSβ subunits of *Homo sapiens* , *Bos Taurus* and*Sus scorfa* have the negatively charged gatekeeper residues- Glu and Asp. The weblogos demonstrated that the most common gatekeeper residues amongst the ADP-forming SCSβ subunits are Asp and Tyr (**Fig. 1b**), while in the GDP-forming SCSβ subunits, most frequently present gatekeeper residues are Glu and Asp (**Fig. 1c**). From the MSA, we have designed various gatekeeper mutants of the*Pf* SCSβ subunit, particularly to alter the charge at the gatekeeper region (**Table 2**).

The molecular models of *Pf* SCSβ subunits from wild-type and various gatekeeper mutants were generated and further electrostatic surfaces were constructed for all the models. The snapshots of the gatekeeper region of the *Pf* SCSβ subunits are represented in**Fig. 2** . The *Pf* SCSβ WT-DY carried the negative and hydrophobic gatekeeper residues (Asp and Tyr) and hence the corresponding gatekeeper region represents the polar character (**Fig. 2A**). The GM-1 KY and GM-2 KK were constructed by sequential substitutions of Asp-Lys and Tyr-Lys, respectively, which are indicated by the presence of positive charge at the gatekeeper region (**Fig. 2B & 3C**). Other gatekeeper mutants- GM-3 DE & GM-4 ED, both carried the negative gatekeeper residues, whereas it is only the latter which emulated the negatively charged Glu and Asp from pig SCSβ subunit (**Fig. 2D & 2E**). Interestingly, the gatekeeper region did not show the negatively charged gatekeeper region as intense as it did in pig SCSβ (**Fig. 2G**) [6].

Determination of the nucleotide specificity of native and recombinant *Pf*SCS enzymes

The nucleotide specificity of *Pf* SCS was determined from the crude lysate of in-vitro cultured *P. falciparum* using the enzymatic assay as described by Hamblin et al. 2008. In accordance with the previous assumption, due to the presence of negative and hydrophobic gatekeeper residues of *E. coli* SCSβ subunit, the *Pf* SCS enzyme should utilize both the nucleotides (ATP & GTP). However, the native *Pf* SCS enzyme was found to be predominantly ADP-forming, having some insignificant activity with the GTP (**Fig. 3**).

Recombinant protein expression was carried out in *E. coli*(BL21DE3) cells for all the *Pf* SCSβ subunits including the wild-type and its various gatekeeper mutants. The affinity chromatography purified fractions of *Pf* SCSβ subunits from the IBs were analyzed by SDS-PAGE (**Fig. 4B-E**) and as mentioned previously, the 6X-His tagged *Blastocystis* SCSα was purified separately in native conditions by affinity chromatography (**Fig. 4A**). The *Pf* SCSβ WT-DY and the *Blastocystis* SCSα subunits were confirmed by western blot showing the presence of two expected size bands by mouse monoclonal anti-His antibody (**Fig. 4F**). Before proceeding for the enzymatic analysis of the recombinant *Pf* SCS, the wild-type and gatekeeper mutants were refolded as described in the methods section.

Enzyme kinetics of *Pf*SCS wild-type and gatekeeper mutant enzymes

It is interesting to note that the *Pf* SCSβ and *Blastocystis*SCSα subunits were separately denatured and refolded into active enzyme confirmations, as per optimized protocols. Since, the nucleotide-binding site lies in the SCSβ subunit, this unique approach was followed after failed attempts to clone the *Pf* SCSα subunit. Interestingly, the*Blastocystis* SCSα subunit did provide the coenzyme A (CoA) binding site essential for the enzyme activity. The refolded wild-type and gatekeeper mutant *Pf* SCS enzymes were subjected to the enzyme kinetics studies. The *Pf* SCS native enzyme was found to be ADP-forming (0.36 μM/min), while a moderate GDP-forming activity (0.10 μM/min) was also observed. However, the enzyme kinetics analysis of the recombinantly expressed *Pf* SCS WT-DY enzyme demonstrated specifically ATP affinity with $\mathbf{K_{\mu ATP}}$ **= 48.46 μM**(**Fig. 5A**) and no activity with the GTP. The positively charged gatekeeper region of the mutant (GM-2 KK) emulated the*Blastocystis* SCS wild-type enzyme in terms of its gatekeeper residues (Lys and Lys). The GM-2 KK mutant showed a mild decrease in the ATP affinity with $\mathbf{K_{\mu ATP}}$ **= 61.32 μM**(**Fig. 5B**). To create a negative gatekeeper region, (Tyr-Glu) mutant- GM-3 DE was constructed and the enzyme kinetics analysis was carried out. The $\mathbf{K_{\mu ATP}}$ **= 84.16 μM** (**Fig. 5C**) values again demonstrated the enzyme to be ADP-forming, exclusively, contrary to the case in *Blastocystis* SCS, where the negative gatekeeper region demonstrated dual nucleotide specificity with the introduction of negative gatekeeper residues (Glu and Asp) (Vashisht et al. 2017). In order to further emulate the sequence matched gatekeeper residues from pig SCS, another mutant GM-4 ED with (Glu and Asp) was constructed. A similar

observation with$\mathbf{K_{\mu ATP} = 118.9\ \mu M}$ (**Fig. 5D**) demonstrated only ATP utilizing potential of the enzyme. However, we have recorded some insignificant activity with GTP in case of GM-3 DE and GM-4 ED *Pf* SCS enzymes and thus the K_m values could not be calculated for GTP.

Discussion

In the absence of any biochemical studies on *Pf* SCS enzyme with particular focus on its nucleotide specificity, this study stands right with following novel aspects- **a)** identification of the corresponding gatekeeper residues from phylogenetically diverse organisms, **b)** assessing the substrate specificity of native*Pf* SCS, **c)** refolding of recombinantly expressed SCSβ subunits of *P. falciparum* (wild-type and gatekeeper mutants) and successful refolding in presence of *Blastocystis* SCSα subunit,**d)** performing enzyme kinetics studies of the refolded enzymes with both the nucleotides (ATP & GTP) and **e)** the effect of the charged gatekeeper residues on the nucleotide specificity.

In a computational attempt to identify the gatekeeper residues among the phylogenetically diverse organisms using MSA tools, we observed that the most common gatekeeper residues in the ADP-forming SCS enzymes were Asp and Tyr (*P. falciparum* , *Homo sapiens* , *Bos taurus* ,*Mus musculus* and *Sus scorfa*), while the GDP-forming enzymes possessed Glu and Asp residues (*Homo sapiens* , *Sus scorfa* , *Bos taurus*) (**Table 1**). Interestingly, our previous study (Vashisht et al. 2017), have shown that the ADP-forming*Blastocystis* SCS is unique in having exclusively positively charged gatekeeper residues (Lys and Lys), where alteration of the charges of the gatekeeper region profoundly altered the substrate specificity. However, the *Pf* SCS has distinct gatekeeper residues (Asp and Tyr) matching with others such as *Homo sapiens* ,*Bos taurus* , *Mus musculus* and *Sus scorfa* . A peculiar characteristic of the SCS enzyme, having dual isoforms in one organism (ADP/GDP-forming), is worth investigating, with particular focus on the gatekeeper residues. As evident by the MSA analysis, the ADP-forming SCS enzymes have Asp and Tyr residues, deviating from the GDP-forming SCS in having Glu and Asp, as gatekeeper residues from the same source. This observation strongly points towards an important role of gatekeeper residues in determining the substrate specificity of SCS enzyme. However, the analysis of gatekeeper residues in other organisms is beyond the scope of the present study.

Enzyme activity of native *Pf* SCS demonstrated the predominantly ADP-forming activity, however, a moderate GDP-forming activity was also observed (**Fig. 3**). It is important to note that the assessment of nucleotide specificity from crude *P. falciparum* lysate is not reliable due to the presence of other parasite proteins, DNA/RNA and nucleotides, metabolites, and variety of other ionic components etc. Hence, we performed the enzyme kinetics analysis with the recombinantly expressed and refolded *Pf* SCS and its mutants. Our enzyme kinetics studies have demonstrated that in *Pf* SCS, the alteration of the electrostatic properties of the gatekeeper residues did not affect the nucleotide specificity, as it did in our previous serendipitous model enzyme- *Blastocystis* SCS. Surprisingly, the *Blastocystis*SCS enzyme with the positively charged gatekeeper residues favored ATP, while with the negatively charged gatekeeper residues, it could utilize GTP as well; particularly due to the electrostatic interactions with the approaching substrate. This led us to hypothesize that it could be a general mechanism for determining the substrate specificity in other enzymes too and it can be further exploited as a novel enzyme engineering approach to alter the substrate specificity. However, in the case of *Pf* SCS, the distinct gatekeeper region as depicted in the electrostatic surfaces models of the wild-type and various mutants of SCSβ subunits, as compared to *Blastocystis* SCSβ subunit was observed. The electrostatic interactions of SCS protein with its approaching substrates (nucleotides) could be masked by other neighboring amino acids and hence could be responsible for a moderate reduction in the ATP affinity of the *Pf* SCS enzyme. However, a detailed structural analysis via molecular modeling and simulation studies could provide a clearer picture of the molecular interactions of the gatekeeper region and the approaching nucleotides in *Pf* SCS. A thorough comparison of the ADP/GDP-forming isoforms of SCS from the same organism would also be a fruitful attempt in understanding the molecular basis of substrate specificity for enzymes, which can bind to similar substrates such as ATP/GTP.

Conclusion

The present study concluded that the *Pf* SCS is an ADP-forming isoform of the SCS enzyme and possess the gatekeeper residues which are similar for the ADP-forming SCS of human, bovine and murine representative organisms. Contrary to our initial assumption that charged gatekeeper residues 'alone' could alter the substrate specificity of nucleotide-binding enzymes such as succinyl-CoA synthetase of *P. falciparum* ; our experimental data demonstrated only a mere reduction in ATP affinity across all the mutants of*Pf* SCS enzyme. Thus, our study again points out the unanswered question to pin-point the molecular interactions required for discrimination of similar substrates by the proteins.

Conflict of Interest

Authors declared there is no conflict of interest with content of the present manuscript. All authors reviewed the final version of a manuscript.

Author contributions

Conceived and designed the experiments: KCP. Performed the experiments: KV, PS & SV. Data analysis and manuscript writing: KCP, KV, RKD and NM.

References

1 Fischer E (1894) Einfluss der Configuration auf die Wirkung der Enzyme. *Berichte der Dtsch. Chem. Gesellschaft* **27** , 2985–2993.

2 Koshland DE (1958) Application of a Theory of Enzyme Specificity to Protein Synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **44** , 98–104.

3 Nobeli I, Laskowski R a, Valdar WS & Thornton JM (2001) On the molecular discrimination between adenine and guanine by proteins.*Nucleic Acids Res.* **29** , 4294–309.

4 Basu G, Sivanesan D, Kawabata T & Go N (2004) Electrostatic potential of nucleotide-free protein is sufficient for discrimination between adenine and guanine-specific binding sites. *J. Mol. Biol.***342** , 1053–66.

5 Gilson MK & Honig BH (1987) Calculation of electrostatic potentials in an enzyme active site. *Nature* **330** , 84–86.

6 Hamblin K, Daron M. Standley, Rogers MB, Alexandra Stechmann AJR, Maytum R, And & Giezen M van der (2008) Localization and nucleotide specificity of Blastocystis.pdf. *Mol. Microbiol.* **68** , 1395–1405.

7 Vashisht K, Verma S, Gupta S, Lynn AM, Dixit R, Mishra N, Valecha N, Hamblin KA, Maytum R, Pandey KC & Van Der Giezen M (2017) Engineering Nucleotide Specificity of Succinyl-CoA Synthetase in Blastocystis: The Emerging Role of Gatekeeper Residues. *Biochemistry* **56** , 534–542.

8 van Dooren GG, Stimmler LM & McFadden GI (2006) Metabolic maps and functions of the Plasmodium mitochondrion. *FEMS Microbiol. Rev.***30** , 596–630.

9 Gardner MJ, Hall N, Fung E, et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419** , 10.1038/nature01097.

10 Ke H, Lewis IA, Morrisey JM, McLean KJ, Ganesan SM, Painter HJ, Mather MW, Jacobs-Lorena M, Llinás M & Vaidya AB (2015) Genetic investigation of tricarboxylic acid metabolism during the plasmodium falciparum life cycle. *Cell Rep.* **11** , 164–174.

11 Nagaraj VA, Sundaram B, Varadarajan NM, Subramani PA, Kalappa DM, Ghosh SK & Padmanaban G (2013) Malaria Parasite-Synthesized Heme Is Essential in the Mosquito and Liver Stages and Complements Host Heme in the Blood Stages of Infection. *PLoS Pathog.* **9** , e1003522.

12 Wolodko WT, Fraser ME, James MNG & Bridger WA (1994) The Crystal Structure of Succinyl-CoA Synthetase from Escherichia. *J. Biol. Chem.* **269** , 10883–10890.

13 Fraser ME, James MN, Bridger W a & Wolodko WT (2000) Phosphorylated and dephosphorylated structures of pig heart, GTP-specific succinyl-CoA synthetase. *J. Mol. Biol.* **299** , 1325–39.

14 Kinoshita K & Nakamura H (2004) eF-site and PDBjViewer: database and viewer for protein functional sites. *Bioinformatics* **20** , 1329–1330.

15 Pandey KC, Singh N, Arastu-Kapur S, Bogyo M & Rosenthal PJ (2006) Falstatin, a Cysteine Protease Inhibitor of Plasmodium falciparum, Facilitates Erythrocyte Invasion. *PLOS Pathog.* **2** , e117.

S. No.	Organism	UniProt IDs of Σ˜Σβ	Gatekeeper residues
	P. falciparum	**Q8ILE9**	**DY**
	Blastocystis	**B3FHPO**	**KK**
	E. coli	**B7M5P1**	**PD**
	Toxoplasma gondii	Q1KSE5	DF
	Leishmania major	Q4Q1C4	KG
	Homo sapiens	Q9P2R7	DY
	Arabidopsis thaliana	O82662	ES
	Bos Taurus (Bovine)	Q148D5	DY
	C. elegans	P53588	DF
	H. influenzae	P45101	KD
	Mus musculus (Mouse)	Q9Z2I9	DY
	Mycobacteriaceae	A3Q5P5	PD
	Oryza (Rice)	Q6K9N6	ES
	S. cerevisiae	P53312	KD
	Sus scorfa (Pig)	O97580	DY
	Drosophila melanogaster	Q9VHJ8	NF
	Rattus norvegicus (Rat)	F1LM47	DY
	Homo sapiens	Q96I99	ED
	Sus scorfa **(Pig)**	**P53590**	**ED**
	Bos taurus (Bovine)	Q3MHX5	ED
	Columba livia (Pegion)	Q9YI36	EN

Ταβλε 1: Λιστ οφ ΑΔΠ-φορμινγ ανδ ΓΔΠ-φορμινγ Σ˜Σβ συβυνιτς φρομ αριους οργανισμς ωιτη τηειρ ΥνιΠροτ ΙΔς. Τηε γατεκεεπερ ρεσιδυες ιν βολδ ανδ σηαδεδ ροως ηαε βεεν υσεδ φορ ςομπαρισον ιν πρειους στυδιες αλσο (Vashisht et al. 2017)**.**

Γατεκεεπερ ρεσιδυες οφ *Πφ* Σ˜Σβ (bold & italics)	**Mutations**	**Primers**
Pf SCSβ wild-type *DY* (WT-DY)	No mutation	**FP**: 5'-TAT*GGATCCA*TGGCCCGTTTTAAGAGCC-3' [*BamHI*] **RP**: 5'-ATT*GTCGAC*TTAAAACGAGATGTCTATG-3' [*SalI*]
Gatekeeper mutant-1 KY (GM-1 KY)	D-K at 95 position	**FP:** 5'-TGGTGATAAT*aag*TTAGTAATAAAAGCTC-3' **RP:** 5'-CAAACGTTTTGTAATAATAAAGC-3'

Γατεκεεπερ ρεσιδυες οφ *Πφ* Σ"Σβ (bold & italics)	Mutations	Primers
Gatekeeper mutant-2 KK (GM-2 KK)	Y-K at 164 position	**FP:** 5'-GAACGTTTTT*aag*TAAGAAAAGAAAGATATA 3' **RP:** 5'-ACATATAAATACAGTATTACATTTTTTTC-3'
Gatekeeper mutant-3 DE (GM-3 DE)	Y-E at 164 position	**FP:** 5'-GAACGTTTTT*gag*TAAGAAAAGAAAGATATA 3' **RP:** 5'-ACATATAAATACAGTATTACATTTTTTTC-3'
Gatekeeper mutant-4 ED (GM-4 ED)	D-E at 95 & Y-D at 164 positions, respectively	**FP:** 5'-TGGTGATAAT*gag*TTAGTAATAAAAG-3' **RP:** 5'-CAAACGTTTTGTAATAATAAAGC-3'

Ταβλε 2: Νομενςλατυρε οφ τηε ωιλδ-τψπε ανδ αριους γατεκεεπερ μυταντς οφ *Πφ*Σ"Σβ συβυνιτς ανδ τηε ρεσπεςτιε πριμερ σεχυενςες υσεδ φορ ςλονινγ. Ρεστριςτιον ενζψμες αρε ιταλιςιζεδ ανδ υνδερλινεδ φορ τηε ωιλδ-τψπε *Πφ*Σ"Σβ συβυνιτ.

Figure legends

Φιγ 1: α) Μυλτιπλε σεχυενςε αλιγνμεντ (ΜΣΑ) οφ αριους Σ"Σβ συβυνιτς φρομ πηψλογενετιςαλλψ διερσε οργανισμς (gatekeeper residues are shaded in grey**). Β) Ωεβλογο ρεπρεσεντινγ τηε γατεκεεπερ ρεσιδυες ιν β) ΑΔΠ-φορμινγ Σ"Σβ συβυνιτς· ς) ΓΔΠ-φορμινγ Σ"Σβ συβυνιτς, ινδιςατινγ τηε μοστ ςομμον ρεσιδυες φρομ τηε ρεπρεσεντατιε οργανισμς αλιγνεδ ιν τηε πρειους φιγυρε. (Ηιγηλιγητεδ βψ ρεδ αρροω).**

Φιγ. 2: Σναπσηοτς οφ τηε ελεςτροστατις συρφαςε μοδελς οφ*Πφ*Σ"Σβ γατεκεεπερ ρεγιον. Electrostatic surfaces of gatekeeper region of *Pf* SCSβ subunits indicated by a black oval. (A) Gatekeeper region of **wild-type-DY** ; (B) Gatekeeper region of**GM-1 KY** ; (C) Gatekeeper region of **GM-2 KK** (D) Gatekeeper region of **GM-3 DE** and (E) Gatekeeper region of**GM-4 ED** . Electrostatic surface of the gatekeeper region shown in red color indicates an overall negative charge, blue color indicates positive charge and the purple color indicates the polar character of the residue. The electrostatic surfaces were prepared by using Modeller9V1032 and eF-surf server and visualized in PDBjViewer. For reference, the SCSβ subunits from *E. coli* , Pig and*Blastocystis* are also represented here (F-H) (Vashisht et al. 2017).

Fig. 3: Initial rates of reaction for native *Pf*SCS enzyme. *Pf* SCS enzyme activity with both the nucleotides (ATP & GTP) at 150 μM concentration. The error bars are represented above the individual columns.

Φιγ. 4: ΣΔΣ-ΠΑΓΕ αναλψσις οφ τηε *Βλαστοςψστις* Σ"Σα ανδ*Πφ*Σ"Σβ συβυνιτς. Α) *Blastocystis***SCS α** - Lanes 2 & 3 containing purified fractions at size 33 kDa, **Β)***Pf***Σ"Σβ ΩΤ-ΔΨ** containing purified fractions in lanes 4-6 at size 52 kDa, **C) GM-2 KK** containing purified fractions in lanes 3 & 4 at size 52 kDa, **D) GM-3 DE** containing purified fractions in lanes 3 & 4 at size 52 kDa and **E)GM-4 ED** containing purified fractions in lanes 3 at size 52 kDa. **F)** Western blot of the *Pf* SCSβ WT-DY and*Blastocystis* SCSα subunits detected by anti-His antibody (Protein marker is represented by kDa).

Fig. 5: Enzyme kinetics of *Pf*SCS recombinantly expressed and refolded enzymes with variable concentrations of ATP & GTP. Graphs are showing the initial rates (μM/min) *vs* ATP & GTP concentrations (μM). **(A)** Graph of *Pf* SCS WT-DY;**(B)** GM-2 KK; **(C)** GM-3 DE and (D) GM-4 ED. The K_m values were calculated by Graphpad Prism 5.0.

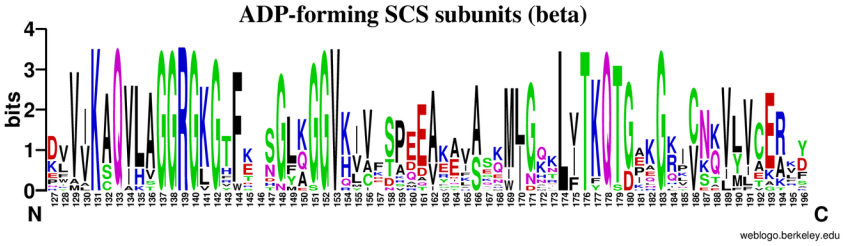
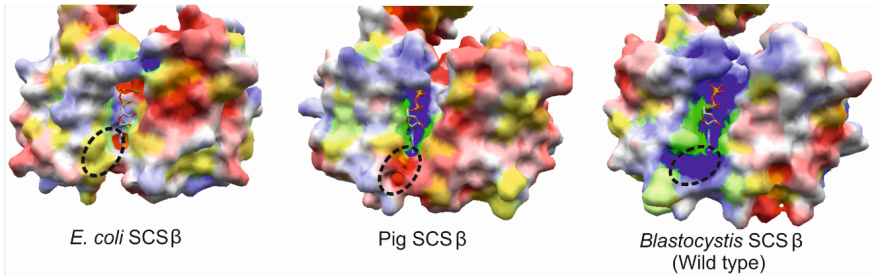


Figure 1



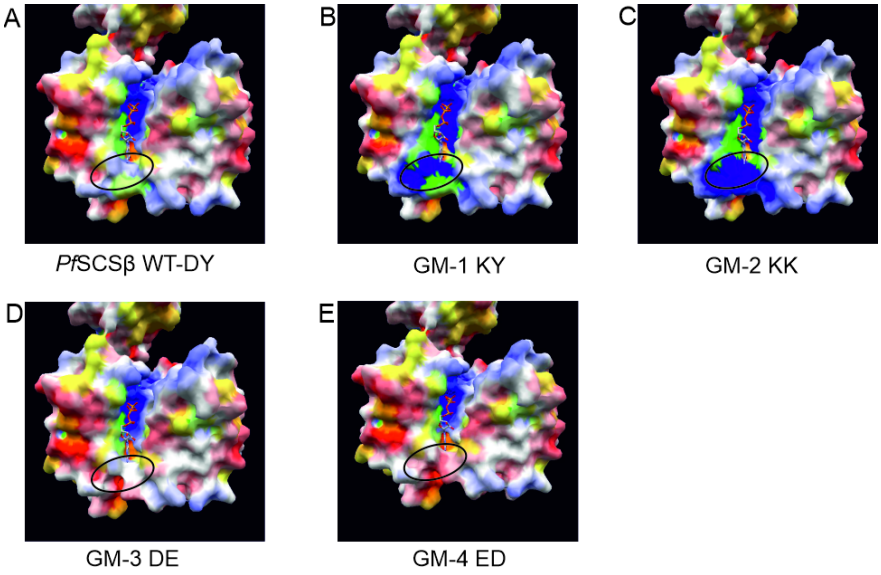


Figure 2

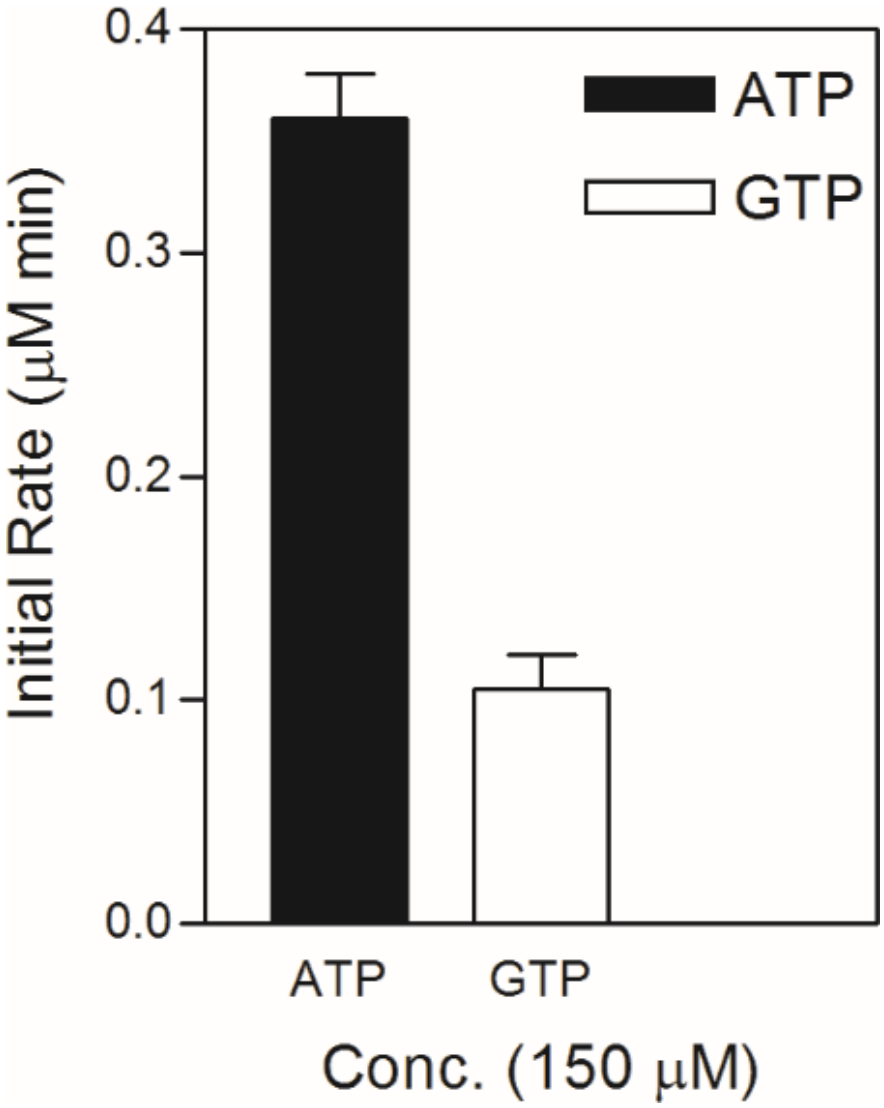


Figure 3

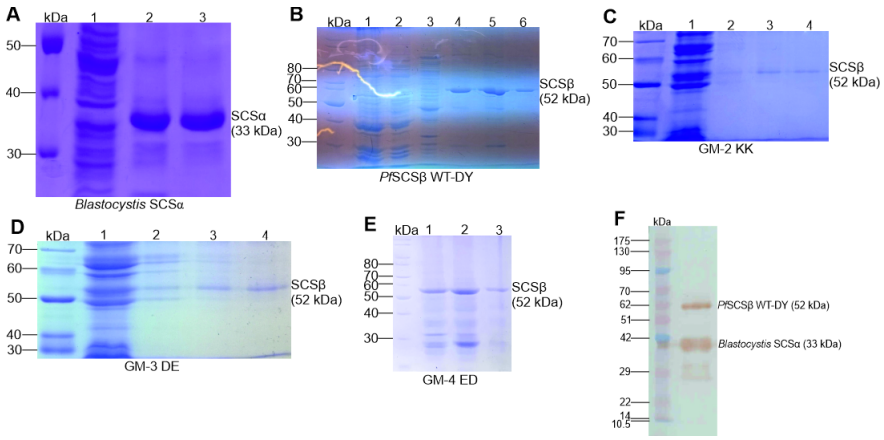


Figure 4

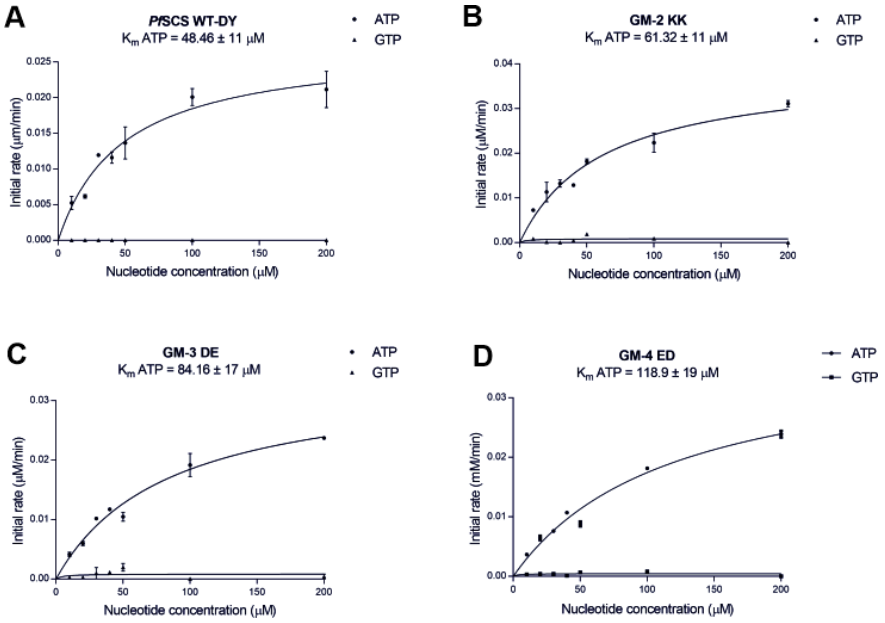


Figure 5