The nucleotide specificity of succinyl-CoA synthetase of Plasmodium falciparum is not determined by charged gatekeeper residues alone

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Keywords
- gatekeeper residues
- malaria
- Plasmodium falciparum
- site-directed mutagenesis
- substrate specificity
- succinyl-CoA synthetase

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(Received 30 April 2020, revised 9 October 2020, accepted 9 November 2020)

doi:10.1002/2211-5463.13034

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] proposes a rigid fit, whereas the ‘induced fit model’ [2] suggests 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 specific amino acids around the substrate play a critical role in determining the substrate specificity of an enzyme [3]. In addition, 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, e.g. adenine and guanine, in the case of nucleotide-binding proteins. Basu et al. [4] reported that a strong ligand-free electrostatic potential could discriminate between A/G binding sites, and hence established the role of an electrostatic component in the molecular discrimination of adenine and guanine. Previously, the electrostatic potential arising from the charged amino acids inside the active site of the 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

Abbreviations
- IB, inclusion body
- MSA, multiple sequence alignment
- PDB, Protein Data Bank
- PfSCS, Plasmodium falciparum succinyl-CoA synthetase
- SCS, succinyl-CoA synthetase
- TCA, tricarboxylic acid
- WT, wild-type
not been investigated thoroughly. In 2008, Hamblin et al. [6] proposed an electrostatic gatekeeper effect, in which the nucleotide access was controlled by the charged amino acids (gatekeeper residues) outside the binding site of the succinyl-CoA synthetase (SCS) of Blastocystis, a human intestinal parasite. 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 Plasmodium falciparum. P. falciparum is an important human parasite that causes malaria, a significant infectious disease, with ~219 million clinical cases and ~0.43 million deaths worldwide (https://www.mmv.org/newsroom/publications/world-malaria-report-2018). The first line of defense for P. falciparum malaria is artemisinin combination therapies. However, the emergence of resistance against artemisinin combination therapies is a matter of great concern, as was with the previous generation of antimalarials, such as chloroquine, sulfadoxine and pyrimethamine. Therefore, a considerable amount of effort is currently being devoted to identify novel drug targets for malaria, simultaneously expanding the fundamental understanding of Plasmodium biology. SCS is a crucial enzyme of the tricarboxylic acid (TCA) cycle, for its unique capability of generating ATP via substrate-level phosphorylation. In P. falciparum, however, the TCA cycle has been suggested to be of limited importance [8], yet the parasite synthesizes all the TCA cycle enzymes [9]. During the asexual growth of the malaria parasite, the absence of any specific phenotypes in AKDH/SCS and ACS/ASDH knockout lines (KOs), indicated metabolic plasticity in the TCA cycle (where KDH represents α-ketoglutarate dehydrogenase, SCSα subunit, and SDH represents SDH flavoprotein subunit) [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]. This study explored the alteration of the charge of the gatekeeper residues and its subsequent effect on the substrate specificity of PfSCS.

### Materials and methods

#### Computational analysis of the SCS subunits

SCS is composed of two subunits, SCSα and 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 (WT) and various mutant PfSCSβ subunits by using modeeler 9v13 (University of California San Francisco, CA, USA), with the following templates, E. coli SCS [Protein Data Bank (PDB): 1CQI] [12] and pig SCS (PDB: 2FP4) [13]. The models were further analyzed by Ramachandran scatterplots and DOPE

| S. No. | Organism | UniProt IDs of SCSβ | Gatekeeper residues |
|-------|----------|---------------------|---------------------|
| 1     | P. falciparum | Q8ILE9 | DY |
| 2     | Blastocystis | B3FHP0 | KK |
| 3     | E. coli | B7M5P1 | PD |
| 4     | Toxoplasma gondii | O1K5E5 | DF |
| 5     | Leishmania major | Q401C4 | KB |
| 6     | Homo sapiens | Q9P2R7 | DY |
| 7     | Aradibopsis thaliana | O82662 | ES |
| 8     | Bos Taurus (bovine) | Q148D5 | DY |
| 9     | C. elegans | P53588 | DF |
| 10    | H. influenzae | P45101 | KD |
| 11    | Mus musculus (mouse) | Q92219 | DY |
| 12    | Mycobacteriaceae | A305P5 | PD |
| 13    | Oryza (rice) | Q6K9N6 | ES |
| 14    | S. cerevisiae | P53312 | KD |
| 15    | Sus scrofa (pig) | O97580 | DY |
| 16    | Drosophila melanogaster | Q9HJ8 | NF |
| 17    | Rattus norvegicus (rat) | F1LM47 | DY |
| 18    | Homo sapiens | Q96999 | ED |
| 19    | Sus scrofa (pig) | P53590 | ED |
| 20    | Bos taurus (bovine) | Q3M9X5 | ED |
| 21    | Columba livia (pigeon) | Q9Y136 | EN |
Table 2. Nomenclature of the WT and various gatekeeper mutants of PfSCSβ subunits and the respective primer sequences used for cloning. Restriction enzymes are italicized and underlined for the WT PfSCSβ subunit. Mutations are represented by underlined lowercase nucleotides (bold and italics) in the respective mutants.

| Gatekeeper residues of PfSCSβ (bold and italics) | Mutations | Primers |
|--------------------------------------------------|-----------|---------|
| PfSCSβ WT DY (WT-DY) | No mutation | FP: 5'-TATGGATCATGGCCTTTGAAGAAGCC-3' [SapI]
| Gatekeeper mutant-1 | D → K at 95 position | RP: 5'-TGGTGATATTGGTATATGAAAGATATGTCG-3' |
| Gatekeeper mutant-2 | Y → K at 164 position | RP: 5'-CAACGTTTTTGAATATGAACG-3' |
| Gatekeeper mutant-3 | Y → E at 164 position | RP: 5'-AACCGTTTTTGAATATGAACG-3' |
| Gatekeeper mutant-4 | D → E at 95 and Y → D at 164 positions, respectively | RP: 5'-CAACGTTTTTGAATATGAACG-3' |

scores. The electrostatic surfaces of the gatekeeper regions were also constructed using eF-surf server and visualized using PDBViewer [14].

**Determination of the nucleotide specificity of native PfSCS enzyme**

The nucleotide specificity of the native PfSCS enzyme was determined from the lysate of the cultured P. falciparum strain 3D7, as described earlier [15]. In brief, 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 ultrasonication of the cultured parasites, centrifuged at 25,000 g for 15 min at 4 °C. The supernatant was collected, and enzymatic assays were performed as described earlier [6]. In brief, the supernatant containing the native P. falciparum SCS was added to the assay buffer [129 µM CoA, 10 mM sodium succinate, 50 mM KCl, 10 mM MgCl2 and 50 mM Tris–HCl (pH 7.4)] with respective nucleotide substrates (ATP and GTP, 150 µM each). The assay recorded the formation of a thioester bond in succinyl-CoA at 232 nm.

**Cloning, recombinant protein expression and refolding of PfSCS**

The PfSCSβ WT subunit was amplified using the primer sequences given in Table 2. The amplified PfSCSβ gene was ligated in expression vector pET28a vector (Novagen, Merck KGaA, Darmstadt, Germany) with 6X His-tag, using 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 PfSCSβ gatekeeper mutants were generated by a commercially available Q5 site-directed mutagenesis kit (New England Biolabs, MA, USA) 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 PfSCSα subunit; hence the Blastocystis SCSα subunit (having >60% identity with PfSCSα) was chosen to generate the refolded PfSCS enzyme.

The protein expression was carried out using standard protocols, optimized in the laboratory [7]. In brief, the overexpression of the cloned PfSCSβ subunit was induced by the addition of 1 mM IPTG after the A values reached 0.4–0.6, and was grown for 4 h postinduction. The bacterial cell pellets were reconstituted in lysis buffer [50 mM NaH2PO4, 10 mM Tris, 500 mM NaCl, 10 mM imidazole (pH 8.0)] and sonicated. Centrifugation at 25,000 g for 30 min at 4 °C yielded the supernatant and cell debris pellet. The pellet was further processed for isolation of inclusion bodies (IBs) containing the 6X-His-tagged PfSCSβ subunits, washed twice with 1M urea and 1% Triton X-100, and finally with 1 M urea alone. The IBs were solubilized in solubilization buffer containing 6 M guanidine hydrochloride and 10 mM Tris–HCl (pH 8.0) overnight. The purification of PfSCSβ was carried out by a custom-packed column with Ni-NTA resin (Nucloecore; Genetix Biotech Asia, Delhi, India) using a fast-process liquid chromatography system, AKTA Prime, FPLC (GE Life Sciences, MA, USA). The elutions were collected from the 200 mM imidazole fractions and analyzed by SDS/PAGE. The PfSCSβ subunit was confirmed by western blotting using a commercially available mouse monoclonal antibody raised against 6X-His-tag (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). As mentioned previously, the Blastocystis SCSα subunit was used at the time of refolding with PfSCSβ subunit [7]. Both the subunits were again denatured in the solubilization buffer and concentrated using 10 kDa cutoff Centronics (Vivaspin). Optimized refolding was performed in buffer [50 mM Tris–HCl, 25% glycerol, 25 mM DTT and 100 µM MgCl2 (pH 7.2)] with rapid dilution (100-fold) of the respective subunits in 1 : 1 ratio and incubated overnight at 4 °C. The refolded PfSCS
enzymes were again concentrated with a 10 kDa cutoff Amicon stirred-cell (Millipore, Merck KGaA, Darmstadt, Germany) and centrifuged at 14 500 r.p.m. for 15 min at 4 °C, to remove precipitated/misfolded proteins, before performing the enzymatic assays.

Enzyme kinetics of the PfSCS (WT and various gatekeeper mutants)

Enzymatic assays were performed with optimized conditions in buffer [10 mM sodium succinate, 50 mM KCl, 10 mM MgCl₂ and 50 mM Tris–HCl (pH 7.4)]. One hundred twenty-nine micromolar CoA and ~30 nm refolded PfSCS enzymes (WT and various gatekeeper mutants) were added in each reaction mix. Varying concentrations of ATP and GTP were used to carry out the enzymatic reaction. The product formation was followed for 10 min with 1-min intervals. A UV-absorbance at 232 nm was recorded in the quartz cuvette of 10-mm path length corresponding to the 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–Menten constant (Kₘ) by using (GRAPHPAD PRISM, CA, USA) 5.0 software.

Results

Sequence and molecular modeling analysis of the various SCSβ subunits

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 SC has Lys and Lys (positively charged) gatekeeper residues, whereas PfSCS has Asp and Tyr (negatively charged and hydrophobic) gatekeeper residues. Another apicomplexan parasite, Toxoplasma gondii, also has the negatively charged and hydrophobic gatekeeper residues (Asp and Phe), but Leishmania major has positive and nonpolar (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 scrofa have the similar gatekeeper residues as P. falciparum (Asp and Tyr); however, the GDP-forming SCSβ subunits of H. sapiens, B. taurus and S. scrofa have the negatively charged gatekeeper residues, Glu and Asp. The weblogos demonstrated that the most common gatekeeper residues among the ADP-forming SCSβ subunits are Asp and Tyr (Fig. 1B), whereas in the GDP-forming SCSβ subunits, the most frequently present gatekeeper residues are Glu and Asp (Fig. 1C). From the MSA, we have designed various gatekeeper mutants of the PfSCSβ subunit, particularly to alter the charge at the gatekeeper region (Table 2).

The molecular models of PfSCSβ subunits from WT and various gatekeeper mutants were generated, and further electrostatic surfaces were constructed for all the models. The snapshots of the gatekeeper region of the PfSCSβ subunits are represented in Fig. 2. The PfSCSβ WT-DY carried the negatively charged and polar gatekeeper residues (Asp and Tyr), and hence the corresponding gatekeeper region represents the negative and polar character (Fig. 2A). E. coli SCSβ subunit displayed the gatekeeper region as negative and nonpolar as a result of Pro and Asp residues at the gatekeeper region (Fig. 2F). 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,C). Other gatekeeper mutants, GM-3 DE and GM-4 ED, both carried the negative gatekeeper residues, whereas it is only the latter that emulated the negatively charged Glu and Asp from the pig SCSβ subunit (Fig. 2D,E). 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 PfSCS enzymes

The nucleotide specificity of PfSCS was determined from the crude lysate of in vitro-cultured P. falciparum using the enzymatic assay, as described by Hamblin et al. [6]. In accordance with the previous assumption, because of the presence of negative and hydrophobic gatekeeper residues of the E. coli SCSβ subunit, the PfSCS enzyme should use both nucleotides (ATP and GTP). However, the native PfSCS 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 PfSCSβ subunits, including the WT and its various gatekeeper mutants. The affinity chromatography-purified fractions of PfSCSβ 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 PfSCSβ WT-DY and the Blastocystis SCSα subunits were confirmed by western blot
Fig. 1. (A) MSA of various SCSβ subunits from phylogenetically diverse organisms (gatekeeper residues are shaded in gray). Weblogo representing the gatekeeper residues in (B) ADP-forming SCSβ subunits and (C) GDP-forming SCSβ subunits, indicating the most common residues from the representative organisms aligned in the previous figure (highlighted by red arrows). The height of the amino acid letter indicates its prevalence in the number of sequences available.
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 PfSCS, the WT and gatekeeper mutants were refolded as described in Materials and methods.

It is interesting to note that the PfSCSβ and Blastocystis SCSα subunits were separately denatured and refolded into active enzyme confirmations, as per optimized protocols. Because the nucleotide-binding site lies in the SCSβ subunit, this unique approach was followed after failed attempts to clone the PfSCSα subunit. Interestingly, the Blastocystis SCSα subunit did provide the CoA binding site essential for the enzyme activity. The refolded WT and gatekeeper mutant PfSCS enzymes were subjected to enzyme kinetics studies. The PfSCS native enzyme was found to be ADP forming (0.36 υM/min), while a moderate GDP-forming activity (0.10 υM/min) was also observed. However, the enzyme kinetics analysis of the recombinantly expressed PfSCS WT-DY enzyme demonstrated specifically ATP affinity with $K_{\text{mATP}} = 48$ υM (Fig. 5A) and no activity with the GTP. The positively charged gatekeeper region of the mutant (GM-2 KK) emulated the Blastocystis SCS WT enzyme in terms of its gatekeeper residues (Lys and Lys). The GM-2 KK mutant showed a mild decrease in the ATP affinity with $K_{\text{mATP}} = 61$ υM (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 $K_{m\text{ATP}} = 84 \, \mu\text{M}$ (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) [7]. To further emulate the sequence-matched gatekeeper residues from pig SCS, we constructed another mutant GM-4 ED with Glu and Asp. A similar observation with $K_{m\text{ATP}} = 119 \, \mu\text{M}$ (Fig. 5D) demonstrated only ATP using the potential of the enzyme. However, we have recorded some insignificant activity with GTP in the case of GM-3 DE and GM-4 ED PfSCS enzymes, and thus the $K_m$ values could not be calculated for GTP.

**Discussion**

In the absence of any biochemical studies on PfSCS enzyme with particular focus on its nucleotide specificity, this study stands right with following novel aspects: (a) identifying the corresponding gatekeeper residues from phylogenetically diverse organisms, (b) assessing the substrate specificity of native PfSCS, (c) refolding of recombinantly expressed SCSβ subunits of *P. falciparum* (WT and gatekeeper mutants) and successful refolding in the presence of the Blastocystis SCSα subunit, (d) performing enzyme kinetics studies of the refolded enzymes with both nucleotides (ATP and GTP), and (e) determining the effect of the charged gatekeeper residues on the nucleotide specificity. However, it is worth mentioning that with the two separate model systems (Blastocystis and *P. falciparum* SCS), where in the case of Blastocystis SCS charged gatekeeper residues were able to discriminate between ATP and GTP, the charged gatekeeper residues of *P. falciparum* altered only the binding affinity of ATP, implying that charged gatekeeper residues might be a way of nucleotide discrimination by proteins, but not a general mechanism for ATP versus GTP discrimination.

In an 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*, *H. sapiens*, *B. taurus*, *M. musculus* and *S. scrofa*), while the GDP-forming enzymes possessed Glu and Asp residues (*H. sapiens*, *S. scrofa* and *B. taurus*) (Table 1). Interestingly, our previous study [7] has 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 PfSCS has distinct gatekeeper residues (Asp and Tyr) matching with others, such as *H. sapiens*, *B. taurus*, *M. musculus* and *S. scrofa*. A peculiar characteristic of the SCS enzyme to have two 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 toward an important role of gatekeeper residues in determining the substrate specificity of the SCS enzyme. However, the analysis of gatekeeper residues in other organisms is beyond the scope of this study.

Enzyme activity of native PfSCS 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, a variety of other ionic components, etc. Hence we performed the enzyme kinetics analysis with the recombinantly expressed and refolded PfSCS and its mutants. To explore a unique aspect in the refolding process of PfSCS, we used the Blastocystis SCSα subunit to refold along with the PfSCSβ subunit. Refolding of the chimeric subunits (SCSα from Blastocystis and SCSβ from *P. falciparum*) to a functional enzyme.

![Fig. 3. Initial rates of reaction for native PfSCS enzyme.](image-url)

**Fig. 3.** Initial rates of reaction for native PfSCS enzyme. PfSCS enzyme activity with both nucleotides (ATP and GTP) at 150 μM concentration (Conc.). The error bars represent the standard error of the mean from duplicate experiments.
successfully validated that swapping of SCS subunits among different organisms is feasible. Because of the nucleotide binding site in the \textit{PfSCS} subunit, it was possible to investigate the nucleotide specificity of the \textit{PfSCS} by the chimeric refolded enzyme. The enzyme kinetics studies have demonstrated that in \textit{PfSCS}, 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, \textit{Blastocystis SCS}. Surprisingly, the \textit{Blastocystis SCS} enzyme with the positively charged gatekeeper residues favored ATP, whereas with the negatively

\textbf{Fig. 4.} SDS/PAGE analysis of the \textit{Blastocystis SCS\textalpha} and \textit{PfSCS\textbeta} subunits. (A) \textit{Blastocystis SCS\textalpha}, lanes 2 and 3 containing purified fractions at size 33 kDa. (B) \textit{PfSCS\textbeta} WT-DY containing purified fractions in lanes 4–6 at size 52 kDa. (C) Gatekeeper mutant GM-2 KK containing purified fractions in lanes 3 and 4 at size 52 kDa. (D) GM-3 DE containing purified fractions in lanes 3 and 4 at size 52 kDa. (E) GM-4 ED containing purified fractions in lane 3 at size 52 kDa. (F) Western blot of the \textit{PfSCS\textbeta} WT-DY and \textit{Blastocystis SCS\textalpha} subunits detected by anti-His antibody (protein marker is represented by kDa).
charged gatekeeper residues, it could use GTP as well, particularly because of 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 as well, 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 WT and various mutants of SCS\( \beta \) subunits, as compared with the \( Blastocystis \) SCS\( \beta \) 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.

**Conclusions**

This study concluded that the \( Pf \)SCS is an ADP-forming isoform of the SCS enzyme and possesses 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 \( Pf \)SCS, 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 pinpoint the molecular interactions required for discrimination of similar substrates by the proteins.
Acknowledgements
The authors thank UGC for financial assistance to KV and Goa University, Goa for providing academic support during his doctoral research program. The study was funded by intramural financial support from ICMR-NIMR, New Delhi (ICMR-NIMR/PB/2011/146).

Conflict of interest
The authors declare no conflict of interest.

Data Accessibility
All the data generated from this study are presented in the manuscript.

Author contributions
KCP and KV conceived and designed the experiments. KV, PS and SV performed the experiments. KCP, KV, RD and NM analyzed the data and wrote the manuscript. All authors reviewed the final version of the manuscript.

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 USA 44, 98–104.
3 Nobeli I, Laskowski RA, Valdar WS and Thornton JM (2001) On the molecular discrimination between adenine and guanine by proteins. Nucleic Acids Res 29, 4294–4309.
4 Basu G, Sivanesan D, Kawabata T and Go N (2004) Electrostatic potential of nucleotide-free protein is sufficient for discrimination between adenine and guanine-specific binding sites. J Mol Biol 342, 1053–1066.
5 Gilson MK and Honig BH (1987) Calculation of electrostatic potentials in an enzyme active site. Nature 330, 84–86.
6 Hamblin K, Standley Daron M, Rogers MB, Alexandra Stechmann AJR, Maytum R and van der Giezen M (2008) Localization and nucleotide specificity of Blastocystis. 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 et al. (2017) Engineering nucleotide specificity of Succinyl-CoA synthetase in blastocystis: the emerging role of gatekeeper residues. Biochemistry 56, 534–542.
8 Van Dooren GG, Stimminger LM and McFadden GI (2006) Metabolic maps and functions of the plasmodium mitochondrion. FEMS Microbiol Rev 30, 596–630.
9 Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419, 498–511.
10 Ke H, Lewis IA, Morrissey JM, McLean KJ, Ganesan SM, Painter HJ, Mather MW, Jacobs-Lorena M, Linás M and 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 and 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 and Bridger WA (1994) The Crystal structure of Succinyl-CoA synthetase from Escherichia. J Biol Chem 269, 10883–10890.
13 Fraser ME, James MN, Bridger WA and Wolodko WT (2000) Phosphorylated and dephosphorylated structures of pig heart, GTP-specific succinyl-CoA synthetase. J Mol Biol 299, 1325–1339.
14 Kinosita K and 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 and Rosenthal PJ (2006) Falstatin, a cysteine protease inhibitor of Plasmodium falciparum, facilitates erythrocyte invasion. PLOS Pathog 2, e117.