Structure and inhibition mechanism of the catalytic domain of human squalene epoxidase

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Squalene epoxidase (SQLE), also known as squalene monooxygenase, catalyzes the stereospecific conversion of squalene to 2,3(S)-oxidosqualene, a key step in cholesterol biosynthesis. SQLE inhibition is targeted for the treatment of hypercholesteremia, cancer, and fungal infections. However, lack of structure-function understanding has hindered further progression of its inhibitors. We have determined the first three-dimensional high-resolution crystal structures of human SQLE catalytic domain with small molecule inhibitors (2.3 Å and 2.5 Å). Comparison with its unliganded state (3.0 Å) reveals conformational rearrangements upon inhibitor binding, thus allowing deeper interpretation of known structure-activity relationships. We use the human SQLE structure to further understand the specificity of terbinafine, an approved agent targeting fungal SQLE, and to provide the structural insights into terbinafine-resistant mutants encountered in the clinic. Collectively, these findings elucidate the structural basis for the specificity of the epoxidation reaction catalyzed by SQLE and enable further rational development of next-generation inhibitors.
The interest in cholesterol has been primarily fueled by the connection of hypercholesterolemia to coronary heart disease. Statins, inhibitors of the rate controlling biosynthetic step HMG-CoA reductase (HMGCR), have transformed this disease landscape and motivated extensive efforts to identify additional agents capable of modulating cholesterol homeostasis. In turn, the availability of these inhibitors has facilitated the identification of additional disease settings where cholesterol pathway modulation may potentially be used for therapeutic purposes. For example, the widespread use of statins has led to epidemiological observations of lowered cancer incidence. However, the efficacy of cholesterol pathway inhibitors in oncology treatment regimens remains to be demonstrated and progress is likely hindered by the lack of clear patient selection strategies. Interestingly, we recently identified a subset of neuroendocrine tumors with unexpected sensitivity to inhibition of SQLE, a step in the cholesterol biosynthetic pathway. SQLE is a flavin adenine dinucleotide (FAD)-dependent epoxidase that catalyzes stereospecific conversion of non-sterol intermediate squalene to 2,3(S)-oxidosqualene (Fig. 1a), the first oxygenation step in cholesterol synthesis. SQLE is proposed to be the second rate-limiting enzyme of the cholesterol biosynthesis downstream of HMGCR5,6 and, as such, is regulated on multiple levels. First, SQLE is a direct target of SREBP2 transcription factor that regulates the majority of genes in the cholesterol biosynthetic pathway. Second, the N-terminus of SQLE protein appears to contain a cholesterol sensing domain that regulates protosequential degradation of SQLE in a cholesterol-dependent manner by relying on MARCH6, an E3 ubiquitin ligase. Interestingly, unsaturated fatty acids, such as oleate, can stabilize SQLE by interfering with MARCH6-mediated degradation. Collectively, these studies highlight multiple mechanisms regulating SQLE to allow for finely-tuned cholesterol homeostasis. In fungi, SQLE is involved in ergosterol biosynthesis and its inhibitor, terbinafine, is approved for the treatment of specific fungal infections.

Based on a growing understanding of the structural and catalytic properties of FAD monooxygenases, SQLE has been classified as a Group E monooxygenase that requires external NADPH-cytochrome P450 reductase (P450R) as an electron donor for squalene oxidation. Small molecule inhibitors of human SQLE, such as NB-598 and compound-4’ (Cmpd-4’), have been previously reported with IC50 in the range of 10–60 nM (Fig. 1b). However, further improvements of these compounds have been hampered by the lack of structural knowledge and by the absence of detailed understanding of their inhibition mechanism.

Here we report the high-resolution crystal structures of the human SQLE catalytic domain and identify critical conformational rearrangements necessary for inhibitor binding. We use the human SQLE structure to further understand the specificity of terbinafine, an approved therapeutic targeting fungal SQLE, and provide the structural insights into terbinafine-resistant mutants encountered in the clinic. Lastly, we model the SQLE substrate, squalene, into the enzyme active site and elucidate the structural basis for the specificity of the catalyzed epoxidation reaction. Collectively, this work provides a foundation for further development of the next generation of SQLE inhibitors.

**Results**

**Functional characterization of human SQLE.** We developed a robust method for expression and purification of recombinant human SQLE proteins for structural and biophysical studies by truncating N- or C-terminal membrane regions (Methods). Individual recombinant proteins were assessed using a thermal shift assay to evaluate their stability and selection of inhibitors for the crystallography experiments (Supplementary Fig. 1). We observed significant stabilization upon addition of NB-598 and Cmpd-4’ inhibitors (ΔTm, 19.7 °C and 17.6 °C, respectively) to the N-terminally truncated SQLE (118–574), resulting in the selection of this construct for further studies.

To confirm that the construct used in crystallography is enzymatically competent, we compared SQLE biochemical activity in multiple systems and contexts. First, we used the recombinant N-terminally truncated SQLE (118–574). Second, we overexpressed full-length SQLE in Sf9 cells using a baculovirus system and utilized a membrane preparation termed baculosomes, analogous to microsomes, as the source of SQLE protein. Finally, we used human liver microsomes (HLM) which have the advantage of providing endogenous SQLE, but contain a full complement of drug metabolizing P450s that may confound the analysis of inhibitor effects. We also developed a liquid chromatography–mass spectrometry (LC-MS) method to directly measure the product of the SQLE reaction, 2,3-oxidosqualene, which provided increased throughput over previously described thin layer chromatography-based assay system. We compared the activity of SQLE (118–574) with that of full-length SQLE in baculosome preparations and the endogenous SQLE from HLM, and found that affinities for both FAD (5.2 ± 0.5 µM for SQLE (118–574), 8.1 ± 0.6 µM for baculosome SQLE, 9.6 ± 0.5 µM for HLM), and squalene (1.9 ± 0.4 µM for SQLE (118–574), 3.3 ± 0.7 µM for baculosome SQLE, 2.9 ± 0.2 µM for HLM) did not differ significantly among the three systems (Fig. 1c and Table 1). The kcat and kcat/KM for SQLE (118–574) were 2.09 ± 0.12 min−1 and 1.10 ± 0.9 × 106 M−1 min−1, for baculosome SQLE were 1.40 ± 0.3 min−1 and 4.25 ± 0.8 × 106 M−1 min−1, respectively. The kcat and kcat/KM for the HLMs were approximately ten-fold lower at 0.21 ± 0.02 min−1 and 1.38 ± 0.4 × 104 M−1 min−1, possibly due to loss of specific activity in the process of microsome preparation. The kinetic parameters obtained in these studies confirm the robustness of the recombinant proteins and place them well within the range of other metabolic enzymes.

**SQLE structure in complex with inhibitors.** We determined the structure of human SQLE de novo by crystallizing the ternary complex of L-selenomethionine (SeMet)-incorporated protein with Cmpd-4’, FAD and by performing multiwavelength anomalous dispersion experiments to derive the phases used for structure solution (Table 2). The initial model was improved by extensive manual rebuilding and by refining against the higher resolution (2.30 Å) native dataset. The final model consists of SQLE amino acid residues 121–574 with well-defined electron density for the FAD and Cmpd-4’ (SQLE•FAD•Cmpd-4’) (Supplementary Fig. 2a). Using the same crystallization conditions, we also determined the NB-598 complex structure (SQLE•FAD•NB-598) at 2.50 Å resolution using phases derived from the SQLE•FAD•Cmpd-4’ complex (Supplementary Fig. 2b). The overall structure of SQLE exhibits the predicted split domain architecture with FAD and substrate-binding domains interspersed within the primary structure, followed by helical membrane-binding domain at the C-terminus (Fig. 2a, Supplementary Fig. 3). The FAD-binding domain adopts a three-layer β/α sandwich architecture, using the CATH nomenclature, which is alternatively referred to as the GR2 Rossman fold. The substrate-binding domain adopts a two-layer β/α sandwich domain with seven-stranded β-sheet structure, followed by two helices at the C-terminus. While we observed a dimeric SQLE molecule in the asymmetric unit, we speculate that this is a technical artifact, since characterization by size-exclusion chromatography showed it to be a monomer in solution (Supplementary Fig. 4).
The structures clearly show that Cmpd-4′′ and NB-598 bind in a similarly extended conformation in a common site and are surrounded by primarily non-polar residues (Fig. 2b, Supplementary Fig. 5). This site is deeply buried and is located at the interface of the FAD-binding, substrate-binding, and C-terminal helical domains. No significant conformational change was observed between the two X-ray structures which can be superimposed with a root mean square deviation (RMSD) of 0.14 Å. The two inhibitors share multiple structural features, such as N-benzyl-N-ethanamine linker, the nitrogen of the central linker that is connected to aliphatic chains, and the phenyl ring that is connected to mostly aromatic moieties. The aliphatic groups of both compounds bind deep into a helical bundle region toward C-terminus and are surrounded by L469, L473, C491, F495, P505, L508, L509, L519, H522, F523, and V526. The aromatic groups of the inhibitors occupy the open end of the pocket proximal to FAD and are surrounded by the apolar residues F166, Y195, A322, L333, Y335, P415, L416, and G418. The aromatic rings of the compounds do not seem to establish any stacking interactions with the surrounding aromatic residues. The ethanamine linkers in both X-ray structures bind in an area defined by Y195, I197, I208, L234, L416, T417, L473, F477, F495, P505, V506, and L509. Both ligands establish a hydrogen bond between the tertiary amine and the hydroxyl moiety of Y195. This hydrogen bond is the only specific and directional interaction established by both compounds with the SQLE. Consistently, the tertiary amine motif is a common feature in all SQLE inhibitors published to date and the interaction with conserved Y195 explains its required presence18,19.

**Fungal SQLE modeling and terbinafine binding.** Interestingly, terbinafine, an approved agent targeting fungal SQLE, also contains the tertiary amine motif (Fig.3a). There has been a considerable interest in using terbinafine as a tool to explore the consequences of SQLE inhibition in human cancer cell lines20,21. We tested the terbinafine in the HLM assay and determined it to be a weak partial inhibitor with a relative IC50 of 7.7 µM and a maximal inhibition of 65% at 100 µM inhibitor concentration (Fig. 3b). This suggests that terbinafine is not optimal for studying human SQLE, particularly when compared to NB-598 or Cmpd-4′′. Alignment of SQLE from clinically relevant fungal strains, such as Trichophyton rubrum, Trichophyton mentagrophytes, and Candida albicans to mammalian sequences show that compound binding site is highly conserved (Supplementary Fig. 3a).

**Table 1 Kinetic parameters for the SQLE reaction in different experimental models**

| Model                  | FAD $K_M$ (µM) | Squalene $K_M$ (µM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_M$ (M$^{-1}$min$^{-1}$) |
|------------------------|----------------|--------------------|------------------------|-----------------------------------|
| SQLE (118–574)         | 5.2 ± 0.5      | 1.9 ± 0.4          | 2.09 ± 0.12            | 1.10 ± 0.9 × 10^6                 |
| SQLE baculosome        | 8.1 ± 0.6      | 3.3 ± 0.7          | 1.40 ± 0.3             | 4.25 ± 0.8 × 10^5                 |
| HLMs                   | 9.6 ± 0.5      | 2.9 ± 0.2          | 0.21 ± 0.02            | 1.38 ± 0.4 × 10^4                 |

The structures clearly show that Cmpd-4′′ and NB-598 bind in a similarly extended conformation in a common site and are surrounded by primarily non-polar residues (Fig. 2b, Supplementary Fig. 5). This site is deeply buried and is located at the interface of the FAD-binding, substrate-binding, and C-terminal helical domains. No significant conformational change was observed between the two X-ray structures which can be superimposed with a root mean square deviation (RMSD) of 0.14 Å. The two inhibitors share multiple structural features, such as N-benzyl-N-ethanamine linker, the nitrogen of the central linker that is connected to aliphatic chains, and the phenyl ring that is connected to mostly aromatic moieties. The aliphatic groups of both compounds bind deep into a helical bundle region toward C-terminus and are surrounded by L469, L473, C491, F495, P505, L508, L509, L519, H522, F523, and V526. The aromatic groups of the inhibitors occupy the open end of the pocket proximal to FAD and are surrounded by the apolar residues F166, Y195, A322, L333, Y335, P415, L416, and G418. The aromatic rings of the compounds do not seem to establish any stacking interactions with the surrounding aromatic residues. The ethanamine linkers in both X-ray structures bind in an area defined by Y195, I197, I208, L234, L416, T417, L473, F477, F495, P505, V506, and L509. Both ligands establish a hydrogen bond between the tertiary amine and the hydroxyl moiety of Y195. This hydrogen bond is the only specific and directional interaction established by both compounds with the SQLE. Consistently, the tertiary amine motif is a common feature in all SQLE inhibitors published to date and the interaction with conserved Y195 explains its required presence18,19.
### Table 2 X-ray data collection, phasing, and refinement statistics

|                  | SQLE•FAD• Cmpd-4′′ | SeMet-SQLE•FAD• Cmpd-4′′ | SQLE•FAD• NB-598 SQLE•FAD |
|------------------|--------------------|--------------------------|---------------------------|
|                  | Native Peak        | Inflection               | Remote                    |
|                  | PDB:6C6N          | PDB:6C6P                 | PDB:6C6R                  |
| **Data collection** |                    |                          |                           |
| Wavelength (Å)   | 0.97941            | 0.97909                  | 0.97915                   |
| Space group      | P3_21              | P3_21                    | P3_21                     |
| Cell dimensions  |                    |                          |                           |
| a (Å)            | 126.96             | 127.1                    | 127.86                    |
| b (Å)            | 126.36             | 127.1                    | 127.86                    |
| c (Å)            | 166.12             | 165.91                   | 165.09                    |
| α, β, γ (°)      | 90, 90, 120        | 90, 90, 120              | 90, 90, 120               |
| Resolution (Å)   | 35.79–2.30         | 50–2.75                  | 40.57–2.50                |
| Rmerge (%)       | 8.2 (55.5)         | 9.0 (53.5)               | 8.6 (54.1)                |
| I/σIa            | 12.1 (3.0)         | 52.1 (7.7)               | 15.1 (2.5)                |
| Completeness (%) | 98.8 (99.7)        | 100 (100)                | 88.5 (89.3)               |
| Redundancy       | 5.2 (5.3)          | 18.4 (18.8)              | 4.8 (4.7)                 |
| Figure of merit  | 0.24/0.74          |                           | 7.4 (6.1)                 |
| Rfinal (anomalous)| 0.84               |                          |                           |
| **Refinement**   |                    |                          |                           |
| No. reflections  | 68379              | 48318                    | 31143                     |
| Rwork/Rfree (%)  | 18.90/22.00        | 19.31/23.42              | 19.01/24.85               |
| No. of atoms     |                    |                          |                           |
| Protein          | 7058               | 7050                     | 7060                      |
| Ligand           | 401                | 363                      | 315                       |
| Water            | 179                | 170                      | 58                        |
| B-factors (Å²)   |                    |                          |                           |
| Protein          | 53.46              | 59.69                    | 61.32                     |
| Ligand           | 57.33              | 61.35                    | 57.17                     |
| Water            | 47.31              | 55.42                    | 44.68                     |
| R.m.s deviations |                    |                          |                           |
| Bond lengths (Å) | 0.003              | 0.009                    | 0.004                     |
| Bond angles (°)  | 0.58               | 0.99                     | 0.68                      |

*Values in parentheses are for highest-resolution shell

**Fig. 2** Human SQLE structure and inhibition. a Overall structure of SQLE bound to inhibitor NB-598. SQLE protein is shown in ribbon representation with the FAD-binding domain in green, the substrate-binding domain in magenta, and the C-terminal membrane-associated helical domain in orange. FAD (yellow) and NB-598 (cyan) are shown as sticks. Hetero-atoms follow the color scheme of yellow, blue, red, and orange for sulfur, nitrogen, oxygen, and phosphor, respectively. b NB-598 binding site. NB-598 and FAD are in ball-and-stick presentation with atomic color scheme as described above. The residues forming the compound binding site is in line presentation with the color scheme on carbon atoms to match the domain coloring scheme as in panel a with important specific residues shown as sticks. The van der waals (VDW) contact surface of the pocket within the 4.5 Å of NB-598 shown as semi-translucent surface. Hydrogen bond interaction between Y195 and the central amine of NB-598 is shown as black dashed line with the distance labeled.
amino acids (F166, I197, and L324) positioned near the aromatic side of the inhibitor were not conserved between the species (Fig. 3c), while the amino acids near the linker and the aliphatic side were identical between human and fungal SQLE. The aromatic side of terbinafine contains bulkier naphthalene group in the position of benzene linker of NB-598. Modeling the terbinafine using NB-598 template in human SQLE positions the naphthalene group adjacent to bulkier hydrophobic side chains of I197 and L324. These sub-optimal non-polar contacts are consistent with the observed higher IC_{50} values of terbinafine in the HLM enzymatic assay. Interestingly, residues corresponding to I197 and L324 in dermatophyte SQLE are smaller hydrophobic.
valines, likely resulting in optimal interactions with naphthalene consistent with the reported selectivity profile of terbinafinae10.

Several reports have identified strains resistant to terbinafinae treatment with point mutations detected in fungal SQLE (ERG1 gene) in both clinical and non-clinical settings22-28. We mapped the reported resistant point mutations onto the human SQLE sequence and to the SQLE•FAD•NB-598 structure (Fig. 3d, Supplementary Table 1). Remarkably, all the SQLE resistant mutations are in the inhibitor binding pocket. Mutation of these conserved residues in dermatophytes (L326, L473, F477, F492, F495, L508, P505, and H522 of human SQLE) would be predicted to affect the non-polar interactions with the inhibitor resulting in the loss of biochemical potency. Collectively, our structural insights provide a detailed explanation for the weak inhibitory potency of terbinafinae against human SQLE and offer understanding of the previously identified terbinafinae-resistant mutations.

Design of structurally-related inactive inhibitor analogs. To further enable cell biology studies and to demonstrate the specificity of the observed cellular responses after the addition of NB-598 or Cmpd-4’, we designed small changes in the compounds to make structurally-related inactive analogs (ia). The narrow binding pocket observed in the inhibitor-bound structures (Fig. 2b and Supplementary Fig. 5) is consistent with the steep structure-activity relationship previously observed13,27. We made limited changes to the inhibitor structures (thiophene into benzene in NB-598 and ortho-tolyl into meta-tolyl in Cmpd-4’) on the aromatic side that might result in steric clashes to generate two analogs, termed NB-598.ia and Cmpd-4”.ia (Fig. 4a, Supplementary Fig. 6 and Supplementary Methods). Potent inhibitory activity of NB-598 and Cmpd-4”, and the expected lack of activity of the analogs, was confirmed in the biochemical assay (Fig. 4b).

Structure of unliganded SQLE and the substrate-binding model. To elucidate any SQLE conformational changes that may be associated with inhibitor binding, we crystallized and determined the structure of unliganded enzyme (SQLE•FAD) by using phases derived from the SQLE•FAD•Cmpd-4’ complex (Table 2). The overall structure of SQLE•FAD is similar to that of the inhibitor-bound structure (Supplementary Fig. 7). However, the substrate-binding domain exhibits greater displacement (RMSD 0.5 Å) compared to FAD-binding (RMSD 0.3 Å) or the membrane domains (RMSD 0.2 Å) (Fig. 5a). In addition to the overall domain motions, in the unliganded structure the side chain of Y195 adopts a distinct conformation and forms a hydrogen bond with the side-chain amide of Q168 from the FAD binding domain. In this conformation, the two polar side chains are shielded within the non-polar pocket, and the alternate conformation of conserved Y195 is required to form the critical hydrogen bond with the tertiary amine of the inhibitors (Fig. 5a). To further explore the role of Y195 in SQLE catalysis and inhibition, we designed two specific mutations: a conservative Y195F substitution and a more severe Y195A change. Interestingly, both mutations resulted in a >90% loss of catalytic activity suggesting that hydrogen bonding property of side-chain hydroxyl of Y195 to Q168 side chain is critical for maintaining the SQLE activity (Fig. 5b).

The extended shape and length (~25 Å) of the inhibitor binding pocket with an opening adjacent to flavin group, as well as the predominantly non-polar residues lining the pocket, suggest that it represents the substrate-binding site of SQLE. Our attempts to generate well-diffracting crystals of SQLE•FAD complex with squalene or 2,3(S)-oxidosqualene were not successful. Therefore, we modeled squalene binding by conducting a molecular docking experiment on the unliganded SQLE•FAD structure as a template and showed that most of the amino acid residues in the squalene binding pocket overlap with amino acids involved with inhibitor binding (Fig. 5c). The critical role of the conserved residues surrounding this binding site, such as Y195, Y208, Y335, F477, and F523, is supported by detailed studies of rat SQLE, where mutagenesis of equivalent amino acids resulted in loss of catalytic activity28.

Biochemical mechanism of action of SQLE inhibitors. To further understand how NB-598 and Cmpd-4’ exert their effects, we undertook a more detailed biochemical dissection of their mechanism of action. Notably, both NB-598 and Cmpd-4’ exhibited time-dependent inhibition (Fig. 6a), which along with the high potency observed, suggested a slow tight-binding mode of inhibition. Further studies indicated non-competitive inhibition (Fig. 6b), despite crystallographic evidence suggesting that NB-598 and Cmpd-4’ were binding in the active site and would be expected to be competitive. While a previous report in fact described a competitive mechanism of action for NB-59812, our biochemical studies suggest that, once bound in the active site, both NB-598 and Cmpd-4’ are more resistant to displacement by excess substrate. We hypothesize that the observed binding potencies are at the upper limit of the enzyme-inhibitor system and if higher squalene concentrations were feasible to achieve in the biochemical assay, it might be possible to competitively displace the inhibitors. In the absence of published progress curves in the previous work and similar substrate concentrations, it is difficult to fully account for the differences in the observed mechanisms of action. However, the structural insights, such as the domain motions and the Y195 conformational switch, are consistent with the observed tight-binding mechanism of inhibitor action, which can kinetically present as non-competitive inhibition, even upon binding in the active site at the same location as substrate29,30.

Implications for SQLE catalysis. Among the FAD mono-oxygenases that catalyze a variety of diverse reactions, two-component epoxidation catalysis of Class E enzymes is among the least understood31. This class of epoxidases relies on external P450R for the transfer of reduced flavin, an initial step in the reaction. Binding of reduced flavin is followed by its reaction with oxygen to form a highly reactive intermediate C4a-(hydrO)peroxyflavin at the flavin N5 position32. The best studied member of this family is styrene monooxygenase from Pseudomonas putida, but the reported structure lacks FAD, thus preventing further understanding of flavin stabilization and identification of catalytic residues33. Remarkably, analysis of our structures near the flavin N5-C4a atoms, a critical region that controls oxygen transfer, revealed that a side chain hydroxyl group from a conserved Y335 interacting with N5 via a bridging water molecule that is further anchored to the protein by main chain hydrogen bonds to I162 and E165 (Fig. 7). The volume available above the plane of flavin would indicate a face-on oxygenation and C4a-(hydroxy)peroxyflavin intermediate generation. Our model suggests that this Y335 water-bridged interaction may play a critical role in recruitment of reduced flavin and stabilization of this reactive intermediate to maintain catalytic efficiency. Indeed, Y334A mutation of equivalent residue in rat SQLE showed a significant decrease in catalytic efficiency28. The orientation of squalene from our docked model and the placement of the 2,3-vinyl group in a plane above the flavin ring (Fig. 7) is consistent with the requirement for stereo- and regio-specific catalysis for the formation of the product 2,3(S)-oxidosqualene.
Discussion

In summary, we report the de novo structure of SQLE catalytic domain that is captured with a bound FAD cofactor and potent inhibitors. Our analysis of the unliganded structure provides an understanding of the obligatory conformational rearrangements required for inhibitor binding and, as such, lays the foundation for further development of the next generation of SQLE inhibitors. Furthermore, our studies provide a structural explanation for the large therapeutic window observed in the clinical use of terbinafine and enable the interpretation of terbinafine-resistant...
substrate. Cmpd-4 NB-598 study was performed with IC50 apparent (M)Squalene (µM). The IC50 of NB-598 decreases from 120 nM to 30 nM and that of Cmpd-4 decreases from 800 to 69 nM upon increasing incubation time.

Mechanism-of-action study of NB-598 and Cmpd-4. NB-598 study was performed with fixed concentrations of inhibitor at 0, 10, and 40 nM indicating non-competitive inhibition with respect to squalene substrate. Cmpd-4 study was performed with fixed concentrations of inhibitor at 0, 30, 60, and 180 nM indicating non-competitive inhibition with respect to squalene substrate. Points and error bars are the mean and standard deviation of triplicate experiments.

Methods

Expression and purification of recombinant SQLE protein. The cDNA of human SQLE (NCBI accession number: NM_003129.3) was cloned into pET28a expression vector (Supplementary Table 2). Multiple constructs were designed with six unique N-termini (starting at 1, 101, 111, 118, 124, 144) and four C-termini (ending at 488, 518, 543, 574), along with different affinity/solubilization tags expressed in Rosetta (DE3) E. coli (Shanghai Weidi Biotechnology). Soluble proteins were obtained for four of the expression constructs with N- and C-terminal boundaries corresponding to 101–574, 118–574, 101–488, and 118–488 and were used in crystallization trials. The His6-MBP-TEV-SQLE (118–574) construct in pET28a vector yielded crystals for structure determination. A detergent screen performed using thermal shift assay led to the identification of 3-(3-cholamido-propyl)dimethylammonio-1-propanesulfonate (CHAPS) as having the most stabilizing effect. CHAPS detergent was subsequently used for all extraction, purification and crystallization studies of SQLE protein.

The plasmid bearing the sequence encoding SQLE was transferred into Rosetta (DE3) E. coli. Cells were grown at 37 °C and induced with 1 mM isopropyl β-D-thiogalactopyranoside for about 20 h at 15 °C in Luria Broth media. SeMet incorporated SQLE (118–574) was expressed in M9 media supplemented with glucose, vitamins, and amino acids with L-methionine substituted by SeMet. E. coli cell pellets were harvested and resuspended in buffer A (50 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0, 0.5% CHAPS), and lysed two times by using a Microfluidizer (Microfluidics Corp, USA) at 15,000 psi and then subjected to ultracentrifugation at 40,000×g for 1 h. The supernatant was first loaded on a Ni2+ Sepharose FF column (GE Healthcare, USA). The column was washed with buffer A and eluted with buffer B (50 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 8.0, 0.5% CHAPS). Pooled protein sample was desalted with HiPrep 26/10 desalting column equilibrated with buffer B. His6-MBP tag was removed with TEV protease enzyme (w/w, protease to protein 1:40 ratio) by incubation at 4 °C overnight. Protein sample was recovered from the flow-through of the HisTrap HP column (GE Healthcare, USA) with buffer A, pooled and column desalted with buffer C (50 mM Tris, 50 mM NaCl, pH 8.0, 0.5% CHAPS). Pooled protein sample was desalted with HiPrep 26/10 desalting 1 × 53 ml (Sephadex G-25F, GE Healthcare, USA) preequilibrated with buffer A. Protein sample was concentrated and further purified by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, USA) equilibrated with Buffer D. Peak fractions were pooled and concentrated to about 8 mg/mL, flash-frozen in liquid nitrogen and stored at −80 °C for further use. The SeMet-derivatized SQLE protein sample was purified as described above, except for further purification by SEC. Protein was determined to be >95% pure by SDS-PAGE and eluted as a monomer as assessed by SEC. Accurate-Mass Time-of-Flight (TOF) LC-MS (Agilent 6224 TOF LC-MS, USA) analysis was used to confirm the molecular weights of native and SeMet substituted proteins.

Crystallization and X-ray diffraction data collection. The random microseeded matrix-screening34 was performed at 20 °C by the sitting-drop vapor-diffusion
method using seeds of lysozyme crystals for the initial screening. Purified SQLE (118–374) at a concentration of 8 mg/ml was incubated with a 5-fold molar excess of Cmpd-4 or NB-598 at 4 °C overnight. Optimal crystals could be grown by mixing 200 nanoliter (nL) protein-inhibitor complex solution with 180 nL reservoir solution consisting of 0.2 M ammonium sulfate, 0.1 M tri-sodium citrate pH 5.6, 15–18 % (v/v) PEG 4000, 20 nL seeding solution, and 40 nL 0.1 M hexammine cobalt(III) chloride as an additive and equilibrated against 15 % of reservoir solution at 4 °C. Crystals were prepared by the hanging drop method or that of SQLE in about 60 μL well solution using a glass bead. Crystals appeared in 2–3 days and grew to a dimension of 50–150 μm in about a week. Mature crystals were harvested and cryo-protected in reservoir solution supplemented with 25% (w/v) ethylene glycol. Crystals were flash-cooled in liquid nitrogen using a nitrogen gas stream. Data analysis for recombinant SQLE was performed as for baculosomes, with the addition of 1.5 μg/ml recombinant P450R (Sigma-Aldrich). Data from the mechanism-of-action study were globally fit to the model for 1 - non-competitive inhibition using the equation (1).

\[ y = \frac{y_{max}}{[S] + K_M(1 + \frac{I}{I_{50}})} \]  

(1)

LC-MS/MS analysis of SQLE reaction. Samples were dried down under nitrogen and then reconstituted with 70 μL of 0.2 mg/ml BHT in acetone. An aliquot of 7.5 μL was injected into the UPLC-MS/MS system. The instrument setup consisted of an AB Sciex API 6500 Mass Spectrometer (AB Sciex, USA) equipped with a Waters UPLC Acquity (Waters, USA). The UPLC separation was performed on an ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 μm, Waters) at 40 °C. Formic acid in water (0.1%, v/v, mobile phase A) and a mixture of acetonitrile and isopropanol (18:82, v/v, mobile phase B) were employed as the mobile phase. Analysis for recombinant SQLE was performed as for baculosomes, with the addition of 0.1% formic acid, mobile phase B) were employed as the mobile phase. An isocratic elution of 98% mobile phase B was used and run time was 1.5 min. The flow rate of mobile phase was set at 0.6 mL/min. Squalene and 2,3-oxidosqualene were ionized under a positive ion spray mode and detected through the multiple-reaction monitoring of a mass transition pair at m/z 414.4 + 231.0 and 427.4 + 409.5, respectively.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The atomic co-ordinates and structure factors for all complex structures are deposited in the Protein Data Bank (https://www.rcsb.org) with deposition IDs 6C6N for SQLE-FAD+Cmpd4-4’, 6C6P for SQLE-FAD+NB-598, and 6C6R for SQLE-FAD, which will be available upon publication. A reporting summary for this Article is available as a Supplementary Information file. Other data are available from the corresponding author upon reasonable request.

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