Mechanistic Insights into Oxidosqualene Cyclizations through Homology Modeling

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Abstract: 2,3-Oxidosqualene cyclases (OSC) are key enzymes in sterol biosynthesis. They catalyze the stereoselective cyclization and skeletal rearrangement of (3S)-2,3-oxidosqualene to lanosterol in mammals and fungi and to cycloartenol in algae and higher plants. Sequence information and proposed mechanism of 2,3-oxidosqualene cyclases are closely related to those of squalene-hopene cyclases (SHC), which represent functional analogs of OSCs in bacteria. SHCs catalyze the cationic cyclization cascade converting the linear triterpene squalene to fused ring compounds called hopenoids. High stereoselectivity and precision of the skeletal rearrangements has aroused the interest of researchers for nearly half a century, and valuable data on studying mechanistic details in the complex enzyme-catalyzed cyclization cascade has been collected. Today, interest in cyclases is still unbroken, because OSCs became targets for the development of antifungal and hypocholesterolemic drugs. However, due to the large size and membrane-bound nature of OSCs, three-dimensional structural information is still not available, thus preventing a complete understanding of the atomic details of the catalytic mechanism. In this work, we discuss results gained from homology modeling of human OSC based on structural information of SHC from Alicyclobacillus acidocaldarius and propose a structural model of human OSC. The model is in accordance with previously performed experimental studies with mechanism-based suicide inhibitors and mutagenesis experiments with altered activity and product specificity. Structural insight should strongly stimulate structure-based design of antifungal or cholesterol-lowering drugs.


Key words: cation-π interaction; homology modeling; lanosterol synthase; 2,3-oxidosqualene; squalene-hopene cyclase

Introduction

Oxidosqualene cyclases (recommended name lanosterol synthase, E.C. 5.4.99.7) of mammals and fungi catalyze the highly stereoselective cyclization of linear (3S)-2,3-oxidosqualene 1 to an intermediate protosterol cation 2 and subsequent skeletal rearrangement to lanosterol 3 (Fig. 1).

Complexity, efficiency, and high stereoselectivity of cyclase catalyzed reactions make them prime examples for studying multiple enzyme functions and interesting tools in synthetic chemistry. Triterpene cyclization products represent precursors of all hopanoids and steroids, for example, cholesterol, glucocorticoids, estrogens, androgens, and progesterones. Finally, cyclases are of high economical interest as targets in development of antifungal, hypocholesterolemic, and phytotoxic drugs.

The enzyme-catalyzed cyclization of 2,3-oxidosqualene 1 is initiated through electrophilic (protic) epoxide activation. Because 2,3-oxidosqualene is stable in neutral media and in glacial acetic acid at room temperature for about 1 day, a strong acid was supposed to be required for initiation. Site-directed mutagenesis experiments identified a highly conserved aspartic acid as an essential residue for catalysis, which is thought to play the role of the proton donor for epoxide activation. During the subsequent complex cyclization process, starting from a prefolded chair–boat–chair conformation of 2,3-oxidosqualene 1, four C==C double bonds are successively converted to C—C single bonds, leading to the tetracyclic protosterol cation 2 (Fig. 1). Quantum chemical investigations indicate that the replacement of a CC π-bond (65 kcal/mol bond energy) by a CC σ-bond (85 kcal/mol bond energy) is highly exothermic by −20 kcal/mol. Calculations also showed that nearly no activation energy for an intramolecular process with prefolded conformation (Fig. 1) is required and that the addition of a tertiary cation to a tertiary sp2 carbon atom can be expected to occur as a barrierless collapse. Exothermic reactions of conformationally flexible substrates such as 2,3-oxido-
Figure 1. (Left) OSCs catalyze the conversion of 2,3-oxidosqualene 1 to lanosterol 3 via the protosterol cation 2. Initial substrate chair–boat–chair conformation of 2,3-oxidosqualene 1, putative conformations after A-, B-, and C-ring closure (2,3-oxidosqualene cation numbering), and skeletal rearrangement of protosterol cation 2 (lanosterol cation numbering) through 1,2-shifts of hydride and methyl groups are shown. (Right) SHCs catalyze the conversion of squalene 4 to hopene 5 and diplopterol 6. Initial all-chair conformation of squalene 4 and putative conformations after A-, B-, C-, D-, and E-ring closure (squalene cation numbering) are shown.
squalene 1, including reactive cationic intermediates, are difficult to control in solution and often lead to a broad variety of products. In contrast, the cyclase-catalyzed reactions show enormously high structural and stereochemical control.

High product specificity in cyclases is believed to be achieved through several factors: (1) by enforcing substrates to occupy prefolded conformations, (2) by progression of reaction via rigidly held, partially cyclized carbocyclic intermediates, and (3) by stabilization of intermediate carbocations by cation-π interactions of frequently occurring aromatic residues in the active site, thus preventing early truncation of the cyclization cascade by deprotonation or nucleophilic addition of solvent molecules.

The enzyme’s role in subsequent skeletal rearrangement of the protosterol cation 2 through 1,2-hydride and -methyl group migration is considered to be secondary. The enzyme is thought to protect intermediate cations from being quenched or the reaction being truncated by early deprotonation and promote migration of hydride and methyl substituents toward the lanosterol cation. This assumption is supported by observations on nonenzymatic, Lewis-acid-catalyzed cyclization experiments of 2,3-oxidosqualene 1, which also lead to products arising from methyl group migration.

The terminal step of the enzyme catalysis process is the dep- rotonation of the lanosterol cation 2, which is again believed to take place under rigorous steric control of the enzyme.

Although significant improvements have been made in the field of membrane protein expression and crystallization, X-ray structural information on OSC is still lacking. In cases where structure elucidation fails, model building of target proteins by exploiting homologies to proteins of known structure (templates) becomes important.

We present homology modeling studies on human OSC based on a crystal structure of Alicyclobacillus acidocaldarius SHC. Proposed reaction mechanism (Fig. 1) and sequences (Fig. 2) of SHCs are closely related to OSC and therefore imply structural similarity of the two cyclase families. The generated three-dimensional structural information on human OSC is compared to the widely accepted cyclization mechanism via a prefolded chair-boat–chair conformation of 2,3-oxidosqualene 1 and differences to the SH catalyzed cyclization of squalene 4 are identified on a molecular level. Consistency of the model structure with available experimental data on mechanism-based suicide inhibitors and site-directed mutagenesis studies is clearly demonstrated. The three-dimensional structural information on human OSC provides new insight into the cyclization and deprotonation of 2,3-oxidosqualene 1.

The information gained from the model should help researchers to upgrade their knowledge of the nature of OSC-mediated conversion at the atomic level, for example, size and shape of active site, specific hydrophobic and polar interactions responsible for stereochemical control, and should also set the stage for structure-based design of potent OSC inhibitors.

Results and Discussion

Sequence Alignment, Secondary Structure Prediction, and Homology Modeling

A search with the molecular modeling package MOE (settings: 20% identity, Z-score 5, see Methods for details) with the human OSC sequence against the PDB retrieved only structural information on A. acidocaldarius SHC and verifies the template selection.

Pairwise sequence alignment of human OSC and A. acidocaldarius SHC shows 41% similarity and rather low identity of 26% (Fig. 2). Thus, protein modeling can be used to generate a crude overall skeleton of human OSC and interpretation of entire model properties is speculative. Wendt et al. identified 34 residues (His451 as second sphere residue excluded) participating in active site formation of A. acidocaldarius SHC. Exclusively comparing these active site residues leads to increased similarity of 50% and an identity of 38%. Similarity is even higher (65% sequence similarity and 50% sequence identity) if only those residues are aligned that are close to a modeled hopene position in the active site of SHC, while residues forming the bottom (residues close to Trp196) and entrance tunnel are neglected (Fig. 3A). Obviously, sequence similarity decreases from the catalytic Asp at the top of the active site to the bottom of the hydrophobic tunnel. Thus, a superposition of the SHC structure with the OSC model backbone shows high consistency around the catalytic Asp (Fig. 3A). Based on high identity of active site residues, properties like size, shape, and physico-chemical properties of the human OSC active site can be predicted with some confidence. This, in turn, allows verification of the mechanistic hypothesis on a molecular level. Comparison with experimental results on suicide inhibitors and mutagenesis studies should further allow a refinement of the model structure.

Homology modeling of human OSC is based on the crystal structure of A. acidocaldarius SHC. To examine the suitability of A. acidocaldarius SHC as a template structure measuring sequence similarity and identity, secondary structure elements of human OSC were predicted (independent from A. acidocaldarius SHC) using the Web application SSpro and compared to secondary structure elements of SHC. In accordance to A. acidocaldarius SHC, the main predicted secondary structure element in human OSC are α-helices. Helices of human OSC (n > 4) match similar regions like helices of A. acidocaldarius SHC in the sequence alignment of the two cyclases (Fig. 2, helices α1 to α22 as numbered by Wendt et al.). Furthermore, five short β-strands are assigned in the SHC structure (n ≤ 5). For all of them close β-strands (n ≤ 5) in the human OSC sequence are predicted by PSIPRED (Fig. 2). Good agreement in distribution of secondary structure elements gives evidence for similar folding of the two cyclases and further proves that A. acidocaldarius SHC is an appropriate template for homology modeling of human OSC.

To verify the stereochemical quality of the homology model structure of human OSC, geometrical parameters were checked and a 3D profile of the protein was generated.

MOE’s protein report function indicates no insufficiencies in angles and bond lengths for the minimized model structure of human OSC. Ramachandran et al. showed, that due to conformational restrictions, a plot of the peptides φ and ψ angles in protein structures could be used as a quality measure. Generally, three sets of φ, ψ angle ranges are defined (CORE, ALLOWED, GENEROUS) according to statistical occurrence in the PDB. Figure 4 shows a Ramachandran plot of the human OSC model structure. Proline and glycine residues were excluded because of their atypical dihedral angle distributions. As it can be seen from Figure 4, there are eight residues (1.2% of overall human OSC
sequence) not belonging to either the CORE, ALLOWED, or GENEROUS region. Compared to dihedral angle distributions of 121,879 residues from 463 structures extracted from the PDB (Table 1), where 1.3% of all residues adopt disallowed conformations,33 this is an acceptable overall number of residues to be outliers in the model structure. Furthermore, all of the observed eight outliers are solvent-exposed residues and do not participate in formation of the active site. All outliers are situated at inserts or gaps in the sequence alignment of human OSC and A. acidocaldarius SHC. Thus, disallowed $\phi$, $\psi$ conformations result from inaccuracies in the construction algorithms used in homology modeling rather than from bad template structure selection.

3D profiles (Fig. 5) of OSC model and SHC crystal structure show positive average numbers over all residues, indicating side chains to be in a preferable environment. Average 3D–1D scores per residue for the OSC model and SHC crystal structure are 37.9 and 54.0, respectively. The higher score for SHC, however, indicates a better overall packing of residues for the crystal structure.

![Figure 2. Sequence alignment of Homo sapiens OSC (Hs), Arabidopsis thaliana cycloartenol synthase (At), Saccharomyces cerevisiae OSC (Sc), and Alicyclobacillus acidocaldarius SHC (Aa). Predicted secondary structure elements of human OSC and secondary structure elements of A. acidocaldarius SHC are included. Red rectangles indicate $\alpha$-helices, yellow rectangles $\beta$-sheets. Numbering of $\alpha$-helix and $\beta$-sheet of A. acidocaldarius SHC corresponds to Wendt et al.31](image)

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Figure 3. (A) Superposition of ribbons from *A. acidocaldarius* SHC crystal structure (bold gray) and modeled human OSC (thin red) structure. Atoms of 2,3-oxidosqualene after A-ring closure with calculated Conolly surface are included to show the location of the hydrophobic tunnel. Atoms of the catalytic Asp455 at the top of the cavity and of Trp196 at the bottom of the cavity (human OSC numbering) are also included. All sequence inserts and gaps with \( n \geq 2 \) are located at solvent-exposed positions. High consistency in superposition of backbones can be observed around the catalytic Asp455. (B) Atoms of the catalytic Asp455 and 2,3-oxidosqualene (C6 cation) in prefolded chair–boat–chair conformation. The methyl group at C10 is indicated by a larger ball. The empty sphere indicates the C10 methyl group position for hypothetical all-chair conformation of the substrate. Superposition of aligned ribbon fragments I and II show reversed steric properties of the two cyclases enforcing 2,3-oxidosqualene in OSC catalysis in a chair–boat–chair conformation. (C) Superposition of SHC (gray) and OSC (red) ribbon fragments stabilizing cations after D-ring formation. In *A. acidocaldarius* SHC Phe601 and Phe605 stabilize the tertiary cation at C19 after Markovnikov D-ring closure of squalene 4 and promote ring expansion to form six-membered D-ring including a secondary cation at C18. Aromatic side chains are in an appropriate position to stabilize a cation at C18. In human OSC, an appropriately positioned \( \pi \)-density of aromatic side chains is lacking and rearrangement of the shown protosterol cation C20 (2 in Fig. 1) to a six-membered D-ring is not observed.
processes, for example, protonation and A-ring closure. Discussed separately, although there exists evidence for concerted protonation, individual cyclization steps, and deprotonation are witnessed by comparing the interactions of reaction intermediates with the enzyme. Major reaction steps like following by a single-point mutation of Cys533 (Cys540Ala of \textit{S. cerevisiae}).

Based on crystallographic data \textsuperscript{28} it was proposed that Cys456 and Thr502 residues (\textit{S. pombe} shows conservative substitutions). Acidity of the catalytic Asp might also be increased by hydrogen bonding to ordered water molecules, like it was observed for \textit{A. acidocaldarius} \textit{SHC}. \textsuperscript{28} The crystal structure shows a small cavity close to the catalytic Asp, which is separated from solvent-exposed surface by hydrogen bonding between Arg582 and Glu454. The same motif can be observed in the human OSC model structure. Furthermore, anchimeric assistance to C—O cleavage by proximate and optimally oriented π-nucleophilic double bonds and stabilization of intermediate cations by a high π-electron density or aromatic residues in the active site, like it is observed for a manually modeled prefolded chair–boat–chair conformation of 2,3-oxidosqualene \textbf{1} in the human OSC model, can contribute to increased acidity of the catalytic Asp. Thus, the model on human OSC explains how acidity of catalytic Asp455 is increased and activation barrier of epoxide protonation is reduced to a level, allowing an enzyme aspartic acid to initiate the cyclization cascade by protonation.

Experimental \textsuperscript{34,36–41} and theoretical \textsuperscript{11,34} investigations demonstrated a rapid catalytic cyclization to be promoted not only by an optimally placed, highly acidic Asp residue but also by an optimally prefolded conformation of the substrate. Based on experimental and theoretical studies it is widely accepted that initiation by protonation and A-ring formation represents a concerted process.

Prefolded conformations of the first ring (chair) and first cationic intermediates (tertiary cation) of closed A-rings are identical for substrates of both cyclases (Fig. 1). Positive charges are located above the molecular plain of squalene and 2,3-oxidosqualene \textbf{1}. In accordance with this high consistency in mechanisms, 8 out of 10 residues participating in formation of the active site close to the A-ring are conserved. Only two of the residues are substituted: SHC Asp374 is changed to human OSC Val453 and SHC Asp377 to human OSC Cys456. As has been mentioned previously, Cys456 is supposed to be one of the catalytic Asp activating residues in human OSC. Conserved aromatic residues Phe444, Tyr503, Trp581, and Tyr704 (OSC numbering) stabilize the first tertiary cation at C6. Because there are no gaps or inserts in the sequence alignment close to those residues (Fig. 2), side chains in the model structure of human OSC show similar positions and orientations like those in the SHC crystal structure.

The first mechanistic differences in squalene and 2,3-oxidosqualene \textbf{1} cyclization occur in B-ring formations. Squalene 4 must...

**Table 1. Statistical Occurrence of \( \phi, \psi \) Angles in the PDB**

<table>
<thead>
<tr>
<th>Region</th>
<th>av. PDB (%)</th>
<th>OSC Model (%)</th>
</tr>
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<tbody>
<tr>
<td>Core</td>
<td>81.9</td>
<td>73.2</td>
</tr>
<tr>
<td>Allowed</td>
<td>14.8</td>
<td>22.6</td>
</tr>
<tr>
<td>Generous</td>
<td>2.0</td>
<td>3.0</td>
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<tr>
<td>Outside</td>
<td>1.3</td>
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adopt a chair-like prefolded conformation of the B-ring chain and a tertiary cation after B-ring closure at C10, which is homologous to the previous cation at C6. The methyl groups at C6 and C10 are located above the molecular plain of squalene 4 (Fig. 1). In contrast, 2,3-oxidosqualene 1 reacts from an energetically unfavored boat conformation. Its tertiary cation at C10 and the corresponding methyl group are below the molecular plain of 2,3-oxidosqualene 1 (Fig. 1).

The homology model of human OSC allows an analysis of steric and electronic effects that lead to the observed mechanistic differences. Two fragments in the alignment of SHC and OSC have to be considered in detail:

\[
\begin{array}{c|c|c}
\text{A.a. SHC:} & \text{GG-I} & \cdots \text{GTGF} \\
\text{H.s. OSC:} & \text{TKSI} & \cdots \text{GV-F} \\
\end{array}
\]

Residues Gly259, Gly260, and Ile261 of \textit{A. acidocaldarius} SHC participate in the formation of a loop in the active site above the molecular plain of squalene and are close to the C10 cation and the methyl group at C10 in the prefolded all-chair conformation of squalene 4 (Fig. 3B). The four residues Gly598 –Phe601 form the opposing part of the active site below the molecular plain of squalene 4 in SHC.

Obviously, a positively charged Lys is present above the molecular plain of 2,3-oxidosqualene 1 in the OSC active site. Thus, cation location above the molecular plain of 2,3-oxidosqualene would lead to unfavorable electrostatic interactions with the positively charged Lys331 side chain. C10 cation location below the molecular plain, however, can be well stabilized by \( \pi \)-stacking with side chain of residue Trp581 in human OSC. It is also obvious that there is a reversion in steric properties between the two cyclases. Human OSC has an inserted amino acid above and a deleted residue below the molecular plain of 2,3-oxidosqualene, thus preventing the methyl group at C10 to be located above the molecular plain and avoiding the substrate to take the energetically favored chair conformation. Conversely, some additional space for the methyl group at C10 is offered below the molecular plain through a single gap in the OSC sequence.

The importance of forcing the B-ring in a prefolded boat conformation and avoiding proximity to Lys331 can be seen from mutagenesis studies performed with SHC. \(^{42}\) A single point mutation Leu607Lys introduces a Lys in the hydrophobic tunnel of \textit{A. acidocaldarius} SHC. The position of the Lys607 side chain is close to prefolded the B- and C-ring of squalene. This introduction of a positively charged residue in \textit{A. acidocaldarius} SHC and proximity of the positive charge to intermediate cations truncates the cyclization cascade after B-ring formation and the cation is deprotonated to lead to the bicyclic product \( \gamma \)-polypodatetraene 7 (Fig. 6).

To summarize this step, the model structure of human OSC gives detailed insight into electronic and steric pressure of the enzyme to enforce specific, energetically unfavored (boat vs. chair) substrate conformations to avoid truncation of cyclization by early deprotonation.

\[7\]

\[8\]

**Figure 6.** \textit{A. acidocaldarius} SHC single mutant Leu607Lys leads to formation of \( \gamma \)-polypodatetraene 7 and Phe605Lys to 17-isodammara-20(21),24-diene 8.
For C-ring formation, fragments of both OSC and SHC substrates must adopt a prefolded chair conformation (Fig. 1). The secondary cations at C14 are generated in an anti-Markovnikov sense. Cyclization catalysis of substrate analogues provided evidence that C-ring formation occurs through five-membered ring closure (Markovnikov) followed by subsequent ring expansion. Further support for the rearrangement is provided through computational studies.

As in the formation of the B-ring, cation location is the major difference between OSC and SHC mechanisms during this step. In SHC, the cation at C15 is positioned above the molecular plain of squalene, whereas in OSC the cation at C15 is below the molecular plain of the substrate. Because Lys331 in the model structure of human OSC is situated directly above the two subsequent cationic centers at C10 and C15 (Fig. 3B), there is again an electrostatic force to place the cation below the molecular plain of the substrate and to avoid proximity to Lys331. Steric pressure through the enzyme, however, will only play a secondary role for the prefolded C-ring conformation in both enzymes, because it is the energetically favorable chair conformation that is required for C-ring formation.

SHC cyclization proceeds after C-ring closure with formation of a six-membered D-ring. Chemical studies strongly suggest that D-ring cyclization in SHC proceeds through a five-membered D-ring closure to afford a Markovnikov cation followed by ring expansion to yield a six-membered D-ring. The six-membered D-ring includes a secondary cation at C18. For OSC the cyclization cascade ends after formation of the five-membered D-ring to form the tertiary protosterol cation 2 (Fig. 1). Rearrangement to a six-membered ring including a secondary cation is not observed for OSC.

For the structural interpretation of this reaction step one has to consider a fragment of the sequence alignment and resulting effects on the model structure of human OSC (Fig. 3C):

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600
| A.a. SHC: GFPGDF |
| human OSC:  -FNKSC |
| 700 |
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The two residues Phe601 and Phe605 in A. acidocaldarius SHC participate in formation of the active site below the molecular plain of squalene and are close to the secondary cation at C18 in the six-membered D-ring. This cation at C18 is accommodated exactly between the two aromatic Phe side chains, which are in optimal position for efficient π-cation stabilization, which enable intermediate formation of a secondary cation.

The above sequence alignment shows Phe605 to be substituted by Cys700 in human OSC, while Phe601 is conserved (Phe696). But, due to a gap in human OSC in front of Phe696, the orientation of backbone and side chain atoms of the conserved Phe residues in SHC and OSC are different (Fig. 3C). Phe696 is no longer in an appropriate position to stabilize a cation at C18. Thus, the absence of efficient π-cation stabilization by aromatic side chains, caused by substitution of one Phe and a different side chain position for the second Phe, prevent OSC to form a secondary cation at C18 (anti-Markovnikov). Therefore, formation of the tertiary protosterol cation 2 terminates the cyclization cascade in OSC cyclization.

This observation is strongly supported by mutagenesis studies of A. acidocaldarius SHC. The single point mutation Phe605Lys in A. acidocaldarius SHC truncates the cyclization cascade after formation of a five-membered D-ring and the tertiary cation is deprotonated to give 17-isodammara-20(21), 24-diene 8 (Fig. 6). Thus, the absence of aromatic side chain of Phe605 in SHC, as observed in the model OSC (Fig. 3C), prevents direct anti-Markovnikov six-membered D-ring formation or ring-expansion from five-membered D-ring to six-membered D-ring. Both pathways would include a secondary cation at C18 and stabilizing effects of π-stacking through Phe605 are lacking in this SHC mutant.

Skeletal rearrangement through 1,2-shifts of hydride and methyl substituents (Fig. 1) convert the protosterol cation 2 to lanosterol C8 or C9 cation. Intervention of OSC during rearrangement is supposed to be minor since even the nonenzymatic Lewis acid-catalyzed cyclization of 2,3-oxidosqualene affords at least one product arising from methyl group migration. Homology modeling, however, indicates that OSC does indeed play a role in the skeletal rearrangement. Figure 7 shows high π-electron density around intermediate cations at C6 and C10, which are formed after A- and B-ring closure. Conversely, π-electron density around cations at C15 after C- and protosterol cation 2 after D-ring formation is strongly reduced. Therefore, the positive charge migrates during the rearrangement towards high π-electron density from protosterol cation 2 to lanosterol cation at C8 and C9 within the active site of the modeled OSC (Fig. 7).

Thus, the enzyme’s role in skeletal rearrangement is to shift equilibrium between the protosterol cation 2 toward carbocations at the C8 and C9 position of lanosterol cation.

After skeletal rearrangement to C8 and C9 lanosterol cation the cyclization is terminated by proton removal. This deprotonation has to be under rigorous enzymatic control to avoid product scattering on the final reaction step. OSCs catalyze the highly selective deprotonation to form lanosterol 3, which is the thermodynamically most stable deprotonation product (Saitzeff product) with a tetrasubstituted double bond. Other possibilities to deprotonate the C8 and C9 lanosterol cations could lead to formation of kinetic products like 9β,10α-lanosterol 9 and parkeol 10 (Fig. 8), which include trisubstituted double bonds, and also to formation of cycloartenol 11 containing a cyclopropyl ring. Site-directed mutagenesis studies showed that Glu45, which is located at the bottom of the hydrophobic tunnel in A. acidocaldarius SHC participates in the terminal deprotonation step. In human OSC, Gly107 occupies the SHC Glu45 position and no other basic residue is located in the bottom of human OSC to terminate catalysis by deprotonation of protosterol cation 2. The model structure of human OSC shows His232 to be the closest basic residue and to be well positioned to serve as a catalytic base for deprotonation of lanosterol cation.

To summarize the deprotonation step, SHC has a basic residue (Glu45) at the bottom of the hydrophobic tunnel in an appropriate distance to deprotonate the C23 cation after E-ring closure without affording skeletal rearrangement. In contrast, human OSC shows no comparably positioned basic residue to allow direct C20 cation deprotonation of the protosterol cation 2, and therefore, has to support cation location at C8 and C9 of the lanosterol cation rather
than at C20 in the protosterol cation 2. C8 and C9 lanosterol cation positions are proximate to His232, a residue located in the middle of the hydrophobic tunnel.

**Correlation of the OSC Model Structure to Experimental Studies to Verify the Quality of the Model Structure**

OSCs catalyze the stereoselective cyclization and skeletal rearrangement of (3S)-2,3-oxidosqualene to lanosterol 3 in mammals and fungi. Their counterpart in algae and higher plants is cycloartenol synthase (EC 5.4.99.8), which catalyzes conversion to cycloartenol 11 (Fig. 8). Cyclization mechanism and skeletal rearrangement is identical for the two enzymes. Differences occur only in the final deprotonation step, which lead to the formation of different products lanosterol 3 and cycloartenol 11 (Fig. 8). As it can be expected from high consistency in mechanisms, global sequence alignment of *A. thaliana* cycloartenol synthase and human OSC shows high similarity 55.2% and an identity of 45.8% (Fig. 2).

Product distribution of *A. thaliana* cycloartenol synthase can be altered from 99% cycloartenol 11 by the wild-type enzyme, to 56% cycloartenol and 24% lanosterol by the Ile481Val single mutant, 0% cycloartenol and 65% lanosterol by the Tyr410Thr single mutant and to 0% cycloartenol and 75% lanosterol by the double mutant Tyr410Thr Ile481Val.26 9β–Δ7–Lanosterol 9 and parkeol 10 are formed in different amounts as side products by all three mutated enzymes. Lanosterol, cycloartenol, 9β–Δ7–Lanosterol and parkeol all represent deprotonation products of lanosterol C8 and C9 cations, thus indicating similar overall mechanisms except the final deprotonation steps. Homology modeling studies provide interpretation of these diverse deprotonation paths. For human OSC, Val453 and Thr380 occupy the corresponding mutated positions in the cycloartenol synthase and are responsible for the observed deprotonation to the thermodynamic product (tetra-substituted double-bond) lanosterol 3. Obviously, increasing the size of one or both of the two residues Val → Ile and Thr → Tyr leads to formation of the kinetic products, cycloartenol 11 (includes cyclopropyl fragment), 9β–Δ7–Lanosterol 9 and parkeol 10 (three-fold substituted double bonds), because corresponding positions for deprotonation are more easily accessible.

According to the homology models, only three active-site residues in human OSC are different from those in *A. thaliana* cycloartenol synthase (Arg361Lys, Tyr410Thr, and Ile481Val). High similarity and identity of active sites is to be expected because of high mechanistic consistency. As mentioned above, the
prefolded chair conformation of the B-ring in human OSC is enforced through sterical and electronical (Lys331) effects. As a proof of concept of this former hypothesis, Arabidopsis thaliana cycloartenol synthase, which also requires the B-ring in an energetically unfavorable chair conformation uses the same motifs—an inserted amino acid above the molecular plain and a deletion below the molecular plain of the substrate compared to the SHC structure. Thus, the methyl group at C10 is sterically enforced to be situated below the molecular plain. Furthermore, like observed in the human OSC model, a positive charge is introduced in the mainly hydrophobic tunnel of the cycloartenol synthase model (Lys331 in human OSC and Arg361 in Arabidopsis thaliana cycloartenol synthase), thus causing the carbocation at C10 to be situated below the molecular plain of 2,3-oxidosqualene and preferring a boat-like conformation of the substrate.

Further mutagenesis studies have been performed with OSC from Saccharomyces cerevisiae. The single mutants Val454Ala and Val454Gly of Saccharomyces cerevisiae OSC cause significant production of the monocyclic triterpenes achilleol and camelliol (Fig. 9). Mutagenesis of two active site residues of cycloartenol synthase changes product specificity and leads also to formation of lanosterol 3, 9βΔ7-lanosterol 9, and parkeol 10.

Studies on mechanism-based suicide inhibitors also provide results capable to verify the human OSC model structure. Mechanistic studies on mechanism-based suicide inhibitors also provide results capable to verify the human OSC model structure. Mechanistic studies on mechanism-based suicide inhibitors also provide results capable to verify the human OSC model structure.
anism-based suicide inhibitors are capable of covalently labeling the enzyme. After digestion and sequencing, labeled residues can be identified as active-site residues, and their position in the active site relative to catalytic Asp at the top of the hydrophobic tunnel can be estimated.

For checking the correct placement of residues in the model structure of human OSC, experimental results from suicide inhibitors (Fig. 12) were compared to the model OSC structure. Tritiated compounds 14, 15, and 16 produce reactive mono- and bicyclic cations and label Tyr510 of S. cerevisiae OSC. Tyr510 corresponds to Tyr503 in human OSC, and is in the model structure located close to the catalytic Asp (Fig. 8). For successive occurring cations in the cyclization cascade the distance to the catalytic Asp, the initiation point of the catalysis, increases. Therefore, early occurring mono- and bicyclic cations of suicide inhibitors should label residues close to catalytic Asp. This is indeed observed for 14, 15, and 16 labeling human OSC corresponding active-site residue Tyr510. Cyclization of 20-oxaoxidosqualene 17 and diene 18 (Fig. 12) are expected to produce reactive cations in the vicinity of the D-ring and therefore label residue further away from the catalytic Asp than, for example, from the previously described labeling of Tyr510. Compounds 17 and 18 label His234 in S. cerevisiae OSC (His232 human OSC). In accordance, His232 in the model OSC structure is further away from catalytic Asp than Tyr510. The assumption that later occurring carbocations during catalyzed cyclization label active-site residues more distant from the catalysis initiation point catalytic Asp is fulfilled by the modeled OSC structure.

**Conclusion and Outlook**

We provided structural information on human OSC gained from homology modeling studies, which is in good accordance with the suggested cyclization and rearrangement mechanism of 2,3-oxidosqualene 1 and to previously performed experimental mutagenesis and mechanism-based suicide inhibitor studies, including studies performed on A. acidocaldarius SHC and on A. thaliana cycloartenol synthase. Furthermore, homology modeling of human OSC allowed detailed insight on molecular level properties of the binding site and provided new aspects to contradictory opinions. Protic epoxide activation through catalytic Asp455 was shown to be supported by close hydrogen bond partners and activation through ordered water molecules seems to be feasible. The activation barrier is further lowered through stabilization of intermediate cations by a high $\pi$-electron density or aromatic residues in the active site. Residues causing steric and electrostatic pressure of the enzyme to force the B-ring in a prefolded boat conformation were identified. Molecular level differences resulting in truncation of cyclization after five-membered D-ring formation for OSC were detected and correlate well with mutagenesis experiments performed on A. acidocaldarius SHC. The role of the enzyme during skeletal rearrangement, which in the past was assumed to be secondary, is obviously to push the cation position to higher $\pi$-electron density of aromatic side chains in the active site of human OSC. Thus, the final lanosterol cation 2 gets in an appropriate position to be deprotonated by His232 and lead to selective formation of lanosterol 3. Mutagenesis studies of those active site residues, which were identified to be responsible for specific tasks in differentiating between squalene and 2,3-oxidosqualene cyclization, should strongly be stimulated by now available structural information on human OSC. Knowledge of size, shape, and physico-chemical properties of the binding site of human OSC gained by homology modeling studies offers a new way to apply efficient structure-based drug-design methods on human OSC, aiming to develop more potent antifungal, hypocholesterolemic, and phytotoxic drugs with high economical interest.

Furthermore, the present article provides detailed experiences gained in homology model building, quality estimation, and refinement of model structure and also examination and inclusion of previous experimental work to validate model structures.

**Figure 11.** Orientation of Val453 in human OSC and A-ring in chair conformation of 2,3-oxidosqualene 1. The calculated Conolly surface is included to demonstrate steric fixation of the A-ring through the side chain of Val453.

**Figure 12.** Suicide inhibitors that label OSCs at different residues in the active site.
building will play an important role in the era of exploding amounts of sequence information to support drug discovery research, where detailed structural information on binding sites is required.

Materials and Methods

Hard- and Software

Molecular Modeling studies were performed on a Silicon Graphics Octane R12000 Workstations. MOE 2001.01 (Chemical Computing Group Inc., Montreal, Quebec, Canada) was used for sequence alignment and homology modeling. The quality of the structure was checked by a 3D profile generated with XSAE version 1.5 (Clemens Broger, F. Hoffman–La Roche AG, Switzerland). Manual modeling of substrates was carried out with the modeling package Moloc (Gerber Molecular Design, Basel, Switzerland). Final structures were minimized with Amber 6 (Scripps, UCSF).

Experimental Data Selection

Sequence information on human OSC,48 S. cerevisiae OSC,49 cycloartenol synthase from A. thaliana50 and A. acidocaldarius SHC51 were taken from the Swiss-Prot database (Schulz–Gasch and Stahl • Vol. 24, No. 6 • Journal of Computational Chemistry mean and standard deviation of various measured angles. Morris et al.33 have shown that these quantities are significantly correlated to the resolution at which a protein structure was solved.

The homology models of human OSC and cycloartenol synthase were verified for correct φ vs. ψ angles of amino acid residues.33 A 3D profile was generated using the Roche in-house tool XSAE. A residue-wise list of 1D3D profile scores is generated. A large positive number indicates that a residue is in its preferred environment in terms of solvent exposure and occlusion by polar and hydrophobic atoms. In good structures the average over a window of 21 residues should not become negative.53 However, some residues of model structures showed slightly negative average numbers, mainly due to buried side chains of arginine and lysine at the protein surface. Manual reorientation of side chains to solvent-exposed surface led to positive numbers throughout the model.

Minimization

Resulting model structures were refined by energy minimization using the AMBER6.0 all-atom force field. Minimizations were carried out for 20 iterations of simplex minimization followed by 1500 steps of conjugate gradient minimization.54

Manual Modeling of Substrates

C8 and C9 lanosterol cations, protosterol cation, and lanosterol were manually modeled in the active site of homology models of human OSC and cycloartenol synthase from A. thaliana using the modeling package Moloc.

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