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Site-directed mutagenesis of conserved aromatic residues in rat squalene epoxidase

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Abstract

Squalene epoxidase catalyzes the conversion of squalene to (3*S*)2,3-oxidosqualene, which is a rate-limiting step of the cholesterol biogenesis. To evaluate the importance of conserved aromatic residues, 15 alanine-substituted mutants were constructed and tested for the enzyme activity. Except F203A, all the mutants significantly lost the enzyme activity, confirming the importance of the residues, either for correct folding of the protein, or for the catalytic machinery of the enzyme. Further, interestingly, F223A mutant no longer accepted (3*S*)2,3-oxidosqualene as a substrate, while Y473A mutant converted (3*S*)2,3-oxidosqualene to (3*S*,22*S*)2,3:22,23-dioxidosqualene twice more efficiently than wild-type enzyme. It is remarkable that the single amino acid replacement yielded mutants with altered substrate and product specificities. These aromatic residues are likely to be located at the substrate-binding domain of the active-site, and control the stereochemical course of the enzyme reaction.

Keywords: Squalene epoxidase; Active site; Site-directed mutagenesis; Substrate specificity; Biosynthesis

Squalene epoxidase (SE) (EC 1.14.99.7) is a non-metallic, flavoprotein monooxygenase that catalyzes the conversion of squalene to (3*S*)2,3-oxidosqualene, which is a rate-limiting step of the cholesterol biogenesis (Fig. 1) [1–3]. In addition to oxygen, the enzyme requires FAD, NADPH, NADPH-cytochrome P-450 reductase (EC 1.6.2.4), and a supernatant protein factor [4,5]. Notably, SE is the only known non-cytochrome P-450 enzyme that epoxidizes an unactivated alkene, selecting only a single face of one of six trisubstituted olefins [1]. The reduced form of the flavoprotein would initially react with molecular oxygen by a one-electron transfer to produce superoxide anion, which leads to formation of flavin C(4a)-hydroperoxide that can now act as an electrophile. Oxygen transfer to the terminal C–C double bond of the squalene molecule then takes place to produce (3*S*)2,3-oxidosqualene (Fig. 1A). For the regio- and stereo-specific epoxidation reaction, precise molecular interactions are required for the enzyme–substrate complexes.

The unstable, membrane-bound enzyme with molecular mass of 64 kDa has been purified, cloned, and sequenced from several sources including rat [6] and human [7] (Fig. 2). Further, a truncated recombinant rat SE (Glu^{100}–His^{573}) (Δ^{99}His) without the N-terminal putative membrane-binding domain has been constructed and functionally expressed in *Escherichia coli* [8]. Although the three-dimensional structure of the enzyme is not yet available, recent photoaffinity labeling and site-directed mutagenesis experiments have begun to reveal the active-site residues of the enzyme (*e.g.* Lys399, Arg400, Asp407, Asp426, Lys428, and a possible disulfide bond between Cys490 and Cys557 in rat SE) [9,10]. In addition, highly conserved putative FAD-binding domains; the dinucleotide-binding GXGXXG (β1-sheet-α-helix-β2-sheet) motif, and DG and GD motif, are present in both vertebrate and fungal SEs (Fig. 2) [1].

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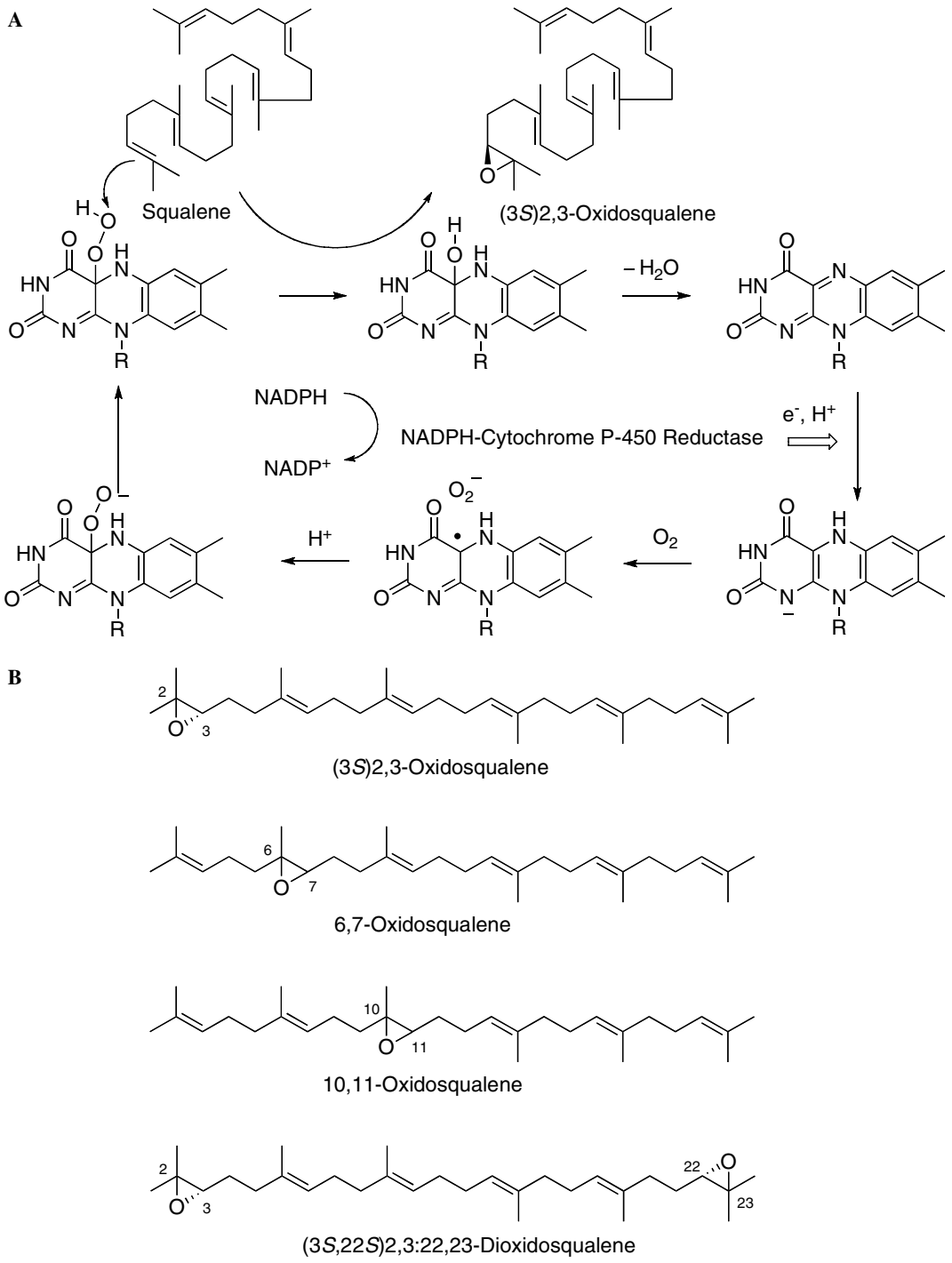

Fig. 1. (A) Proposed mechanism of regio- and stereo-specific epoxidation of squalene to (3*S*)2,3-oxidosqualene by rat SE. (B) Structures of oxidosqualenes and 2,3:22,23-dioxidosqualene.

Interestingly, comparison of the primary sequences of vertebrate and fungal SEs revealed that unusually high numbers of aromatic residues are conserved in the enzyme (Fig. 2). This suggests their functional role at the active site of the enzyme, presumably for the correct folding of the π-electron rich, hydrophobic substrate through π–π interactions to rigidly control the stereochemistry of the enzyme reaction to regio- and stereo-specifically produce (3*S*)2,3-oxidosqualene. To evaluate the importance of the conserved aromatic residues, here we constructed a set of 15 alanine-substituted mutants (Y194A, Y209A, F223A, F228A, F287A, F305A, Y334A, F375A, Y473A, F476A, F491A, Y493A, F522A, F523A, and Y528A) of rat SE, and investigated the effects of the mutagenesis on the enzyme activity and on the stereochemical course of the enzyme reaction.

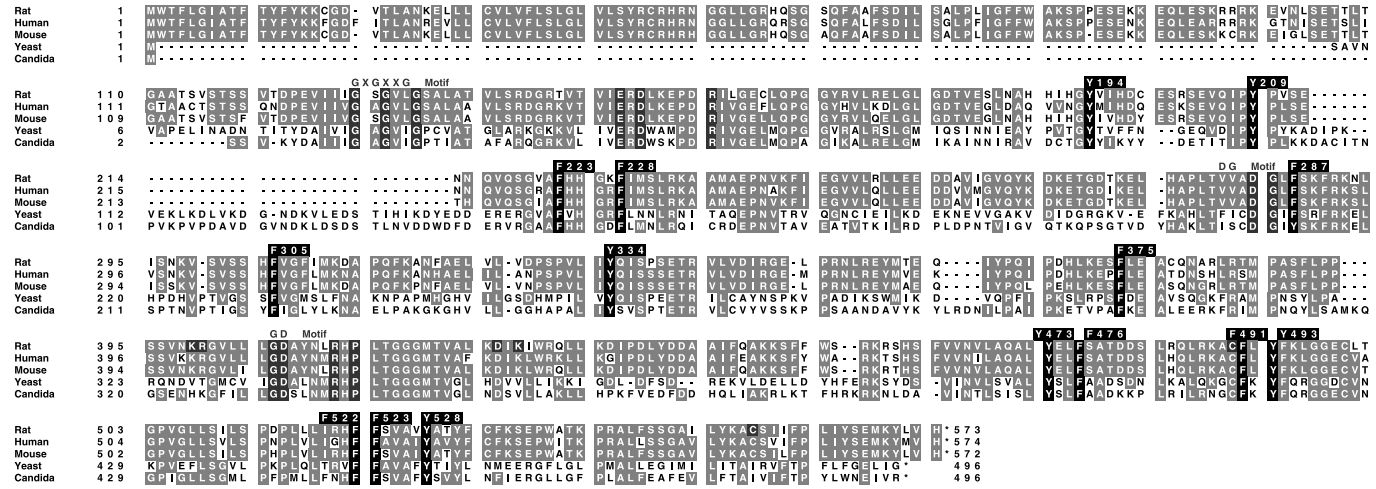

Fig. 2. Comparison of the amino acid sequences of vertebrate and fungal SEs: Rat, *Rattus norvegicus*; Human, *Homo sapiens*; Mouse, *Mus musculus*; Yeast, *Saccharomyces cerevisiae*; Candida, *Candida albicans*. The putative FAD-binding site (the dinucleotide-binding GXGXXG motif, and DG and GD motif) and the conserved aromatic residues are highlighted. Active-site residues (Lys399, Arg400, Asp407, Asp426, Lys428, Cys490, and Cys557) revealed by recent photoaffinity labeling and site-directed mutagenesis [10,11] are also highlighted.

Materials and methods

Chemicals. [1,25-^{14}C]Squalene (57.1 mCi/mmol) was purchased from Chemical Synthetic Facility, University of Utah.

Site-directed mutagenesis. The alanine-scanning mutants of a truncated recombinant rat SE (Glu^{100}–His^{573}) (Δ^{99}His) without the N-terminal putative membrane-binding domain and with an additional hexahistidine tag at the C-termina [8] were constructed (in *Bam*HI/*Nde*I site of pET3a) using the QuickChange™ mutagenesis kit (Stratagene) and a pair of complementary mutagenic primers. Sequence of the forward primer for each mutation were as follows (mutated codons are in italics): Y194A, 5′-GCC CAT CAT ATA CAC GGC *GCC* GTA ATT CAT GAC TGT G-3′; Y209A, 5′-CT GAA GTT CAA ATT CCA *GCC* CCG GTG TCA GAA AAC AAC C-3′; F223A, 5′-CAG AGT GGG GTT GCT *GCC* CAC CAT GGC AAG-3′; F228A, 5′-GCT TTC CAC CAT GGC AAG *GCC* ATC ATG AGT CTC CGG-3′; F287A, 5′-TT GTT GCC GAC GGG CTC *GCC* TCC AAG TTC AGG AA-3′; F305A, 5′-GTC TCT GTT TCC TCC CAC *GCC* GTT GGC TTC ATT ATG-3′; Y334A, 5′-CCC AGT CCA GTT CTC ATC *GCC* CAG ATT TCA CCC AGC-3′; F375A, 5′-GAT CAC TTG AAG GAA TCA *GCT* CTG GAG GCC TGT CAG-3′; Y473A, 5′-GTG CTG GCT CAG GCG TTG *GCT* GAA CTA TTT TCT GC-3′; F476A, 5′-CAG GCG TTG TAT GAA CTA *GCT* TCT GCT ACA GAT GAT TCC-3′; F491A, 5′-G CTC CGA AAA GCT TGC *GCT* CTT TAT TTT AAA CTT GGT GGA G-3′; Y493A, 5′-CAG CTC CGA AAA GCT TGC TTT CTT *GCT* TTT AAA CTT GGT GGA G-3′; F522A, 5′-C CTA TTG ATT CGA CAC *GCC* TTC TCC GTT GCA

Table 1
Steady-state kinetic parameters for enzyme reactions[a]

Enzyme	Squalene			2,3-Oxidosqualene		
	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
WT	4.54 ± 0.47	9.87 ± 2.6	0.460	0.45 ± 0.05	4.25 ± 1.5	0.105
Y194A	0.31 ± 0.02	3.79 ± 1.0	0.082	0.04 ± 0.00	2.19 ± 0.7	0.017
Y209A	0.0057 ± 0.002	0.97 ± 0.5	0.006	NA[b]	NA	NA
F223A	0.40 ± 0.02	0.69 ± 0.2	0.580	NA	NA	NA
F228A	0.0063 ± 0.002	1.91 ± 0.9	0.003	NA	NA	NA
F287A	1.77 ± 0.22	9.66 ± 2.1	0.183	0.32 ± 0.02	2.59 ± 0.5	0.122
F305A	4.14 ± 0.82	25.5 ± 7.3	0.162	0.05 ± 0.01	1.77 ± 0.5	0.030
Y334A	2.67 ± 0.31	12.8 ± 2.7	0.209	0.04 ± 0.01	11.1 ± 4.0	0.003
F375A	NE[c]	NE	NE	NE	NE	NE
Y473A	2.61 ± 0.12	30.1 ± 7.7	0.087	0.72 ± 0.03	3.50 ± 0.5	0.205
F476A	5.91 ± 0.55	22.3 ± 3.4	0.265	0.22 ± 0.03	13.6 ± 3.8	0.016
F491A	3.14 ± 0.67	24.6 ± 8.3	0.128	NA	NA	NA
Y493A	1.10 ± 0.22	10.6 ± 2.4	0.104	0.01 ± 0.00	0.17 ± 0.0	0.066
F522A	0.70 ± 0.03	4.14 ± 0.8	0.169	0.24 ± 0.04	22.5 ± 5.9	0.011
F523A	2.39 ± 0.27	11.36 ± 2.7	0.210	0.31 ± 0.03	10.3 ± 2.3	0.031
Y528A	9.77 ± 2.98	45.3 ± 18	0.216	0.80 ± 0.05	10.4 ± 1.5	0.077

[a] Steady-state kinetic parameters were calculated for either squalene or (*RS*)-2,3-oxidosqualene as a substrate. Lineweaver–Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates ± standard deviation) using EnzFitter software (BIOSOFT).

[b] No activity.

[c] No expression.

GTC-3′; F523A, 5′-C CTA TTG ATT CGA CAC TTC *GC*C TCC GTT GCA GTC-3′; Y528A, 5′-TTC TTC TCC GTT GCA GTC *GC*T GCC ACG TAT TTC TGC-3′.

Enzyme expression and purification. After confirmation of the sequence, the plasmid was transformed into *E. coli* BL21(DE3)pLysS. The cells harboring the plasmid were cultured to an A_{600} of 0.6 in LB medium containing 100 μg/mL of ampicillin at 30 °C. Then, 0.4 mM IPTG was added to induce protein expression. The recombinant enzyme was purified by Ni–NTA agarose and Blue Sepharose CL-6B columns as described before [8]. Protein concentration was determined by the Bradford method (Protein Assay *Dc*, Bio-Rad), using bovine serum albumin as the standard.

Enzyme assay. The reaction mixture contained the recombinant enzyme (1.5 μg/mL), NADPH-cytochrome P-450 reductase (0.05 U), 1 mM NADPH, 0.1 mM FAD, 0.1% Triton X-100, and [1,25-^{14}C]squalene (5 μM, 2×10^4 dpm), in a total volume of 200 μL of 20 mM Tris–HCl, pH 7.4. After incubation at 37 °C for 60 min, the enzyme reaction was quenched by addition of 200 μL of 10% KOH in methanol, and 10 μL of 0.1% cold carrier solution of squalene and oxidosqualene in ethanol. The lipids were then extracted with 400 μL of CH_2Cl_2, and separated by TLC (Whatman silica gel 60A with preadsorband strip) which was developed with 5% ethyl acetate in hexane. The R_f values were 0.84 for squalene and 0.54 for oxidosqualene. Radioactivities were quantified by autoradiography using a bioimaging analyzer BAS-2000II (FUJIFILM). Lineweaver–Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates ± standard deviation) using EnzFitter software (BIOSOFT).

Results and discussion

As described before [11,12], a truncated recombinant rat SE (Glu^{100}–His^{573}) (Δ^{99}His) without the N-terminal putative transmembrane domain, and with an additional hexahistidine tag at the C-terminal for simple purification by Ni-chelate chromatography, was used for the experiments. The recombinant enzyme showed properties very similar to those of native microsomal enzyme with regard to co-factor requirement, pH dependency, and sensitivity to most of known SE enzyme inhibitors [8]. The recombinant wild-type SE showed an apparent $K_M = 9.87$ μM, $k_{cat} = 4.54$ min^{-1}, and $k_{cat}/K_M = 0.460$ min^{-1} μM^{-1} for squalene (Table 1). To evaluate the importance of the conserved aromatic residues in the enzyme, a set of 15 alanine-substituted mutants (Y194A, Y209A, F223A, F228A, F287A, F305A, Y334A, F375A, Y473A, F476A, F491A, Y493A, F522A, F523A, and Y528A) were constructed, and functionally expressed in *E. coli*. As a result, except F375A, all the mutant proteins were expressed in *E. coli* at levels comparable with wild-type enzyme, and purified to homogeneity by a Ni-chelate affinity column.

Out of the 15 mutants, only F223A mutant increased the activity; enzyme kinetics analysis revealed 26% increase in the k_{cat}/K_M value (0.580 min^{-1} μM^{-1}) compared with wild-type SE, while all the other mutants significantly lost the activity and exhibited more than 50% decreases in the k_{cat}/K_M values (Table 1 and Fig. 3). In particular, Y209A and F228A mutants were almost completely inactive, indicating that residues Y209 and F228 are critical for the enzyme activity. On the other hand, F528A mutant exhibited twofold increase in the k_{cat} value (9.77 min^{-1}), but nearly fivefold decrease in the K_M value (45.3 μM). It was thus confirmed that the conserved aromatic residues indeed play important roles in the enzyme, either for the correct folding of the protein, or for the catalytic machinery of the enzyme, presumably for the correct folding of the π-electron rich, hydrophobic substrate squalene through π–π electron interactions. As mentioned, precise molecular interactions of the enzyme–substrate complexes are required to rigidly control the stereochemistry of the epoxidation reaction to regio- and stereo-specifically produce (3*S*)2,3-oxidosqualene. Here it should be noted that formation of either 5,6-oxidosqualene or 10,11-oxidosqualene (Fig. 1B) was not detected in the reaction mixture; the stereochemistry

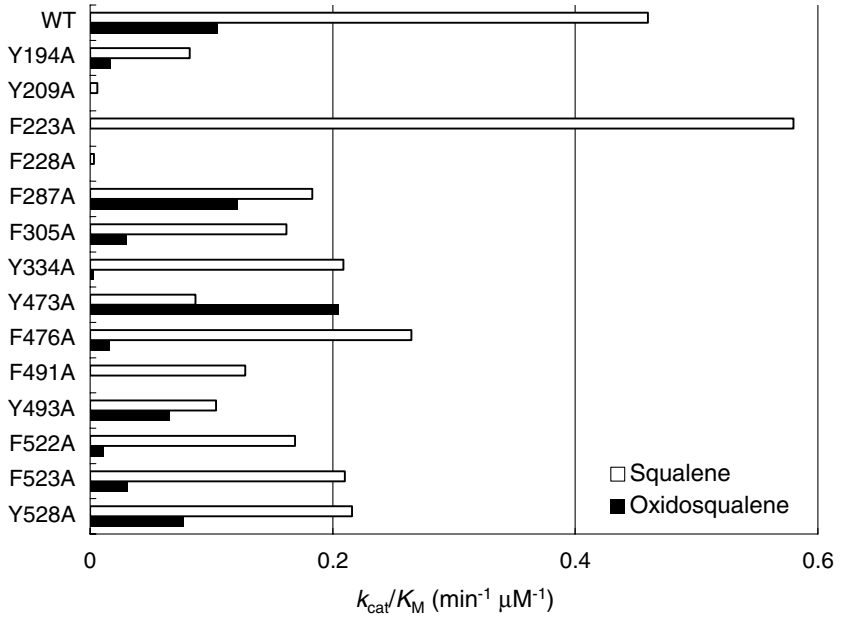

Fig. 3. The k_{cat}/K_M (min^{-1} mM^{-1}) values of recombinant rat SE and the alanine-scanning mutants for squalene (white) and (*RS*)2,3-oxidosqualene (black).

of the epoxidation reaction was thus also strictly controlled in the alanine-substituted mutant enzymes.

SE also catalyzes conversion of (3*S*)2,3-oxidosqualene (OS) to (3*S*,22*S*)-2,3:22,23-dioxidosqualene (DOS) (Fig. 1B), as demonstrated with both partially purified native pig SE [13] and with the recombinant rat SE (Δ^{99}His) [8]. The formation of DOS was reported to be approximately one-half the efficiency of OS [13]. In our assay system, however, the wild-type rat SE exhibited $K_M = 4.25\ \mu M$, $k_{cat} = 0.45\ min^{-1}$, and $k_{cat}/K_M = 0.105\ min^{-1}\ \mu M^{-1}$ for (3*S*)2,3-oxdidosqualene; nearly 80% decrease in the k_{cat}/K_M value, but the K_M value was two times better compared with that for squalene (Table 1 and Fig. 3). Although most of the alanine-substituted SE mutants lost the DOS-forming activity as well, one of the most striking results is that the above mentioned F223A mutant, that produce (3*S*)2,3-oxdidosqualene more efficiently than wild-type SE, almost completely lost the DOS-forming activity; the mutant no longer accepted (3*S*)2,3-oxdidosqualene as a substrate to produce DOS. In contrast, it was remarkable that Y473A mutant produced DOS twice more efficiently than wild-type SE; $K_M = 3.50\ \mu M$, $k_{cat} = 0.72\ min^{-1}$, and $k_{cat}/K_M = 0.205\ min^{-1}\ \mu M^{-1}$ for (3*S*)2,3-oxdidosqualene, while $K_M = 30.1\ \mu M$, $k_{cat} = 2.61\ min^{-1}$, and $k_{cat}/K_M = 0.087\ min^{-1}\ \mu M^{-1}$ for squalene.

It is remarkable that the single amino acid replacement of the conserved aromatic residues of SE, the rate-limiting enzyme of cholesterol biogenesis, yielded mutants with altered substrate and product specificities. Although the three-dimensional structure of the enzyme has not been solved yet, it is likely that these aromatic residues are located at the substrate-binding domain of the active-site of the enzyme, and control the stereochemical course of the enzyme reaction to regio- and stereo-specifically produce (3*S*)2,3-oxidosqualene. Finally, it should be noted that (3*S*,22*S*)-2,3:22,23-dioxidosqualene is further converted to oxysterols such as 24,25-epoxycholesterol, which are thought to regulate the coordinate expression of the genes for HMG-CoA reductase, the LDL-receptor, and other enzymes in the cholesterol biogenesis pathway [14–16]. To fully understand the intimate structural details of the enzyme-catalyzed reaction, further structure function analyses of the enzyme are now in progress in our laboratories.

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