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Kinetic Studies on the Function of All the Conserved Tryptophans Involved Inside and Outside the QW Motifs of Squalene-hopene Cyclase: Stabilizing Effect of the Protein Structure against Thermal Denaturation

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Site-directed mutagenesis experiments were carried out to identify the responsibility of the eight QW motifs for the reaction catalyzed by squalene-hopene cyclase (SHC). Alterations of the conserved tryptophans, which are responsible for the stacking structure with glutamine, into aliphatic amino acids gave a significantly lower temperature for the catalytic optimum as for the mutageneses of QW motifs 4, 5a and 5b, which are specifically present in SHCs. However, there was no change in the optimal temperatures of the mutated SHCs targeted at the other five motifs 1, 2, 3, 5c and 6. Thus, reinforcement against heat denaturation can be proposed as a function of the three QW motifs 4, 5a and 5b, but no function could be identified for the QW motifs 1, 2, 3, 5c and 6, although they are commonly found in all the families of prokaryotic SHCs and eukaryotic oxidosqualene cyclases. On the other hand, the three conserved tryptophans of W169, W312 and W489, which are located inside the putative central cavity and outside the QW motifs, were identified as components of the active sites, but also had a function against thermal denaturation. The other two tryptophan residues of W142 and W558, which are located outside the QW motifs, were found not to be active sites, but also had a role for stabilizing the protein structure. It is noteworthy that the mutants replaced by phenylalanine had higher temperatures for the catalytic optimum than those replaced by aliphatic amino acids. The catalytic optimal pH values for all the mutants remained unchanged with an identical value of 6.0.

Key words: squalene; oxidosqualene; squalene-hopene cyclase; QW motifs; *Alicyclobacillus acidocaldarius*

The conversion of squalene **1** into hop-22(29)-ene **2** and hopan-22-ol **3**, which is catalyzed by squalene-hopene cyclase [EC 5.4.99.-] (SHC), is one of the most intricate biochemical reactions.[1] The cyclization reaction proceeds with a precise enzymatic control to form five rings and nine new stereo-centers (Scheme 1). The polyolefin cyclization reaction of **1** is analogous to that of oxidosqualene into lanosterol and plant triterpenes that is mediated by oxidosqualene cyclases (OSC).[1] Ourisson *et al.*[2-4] have proposed that triterpenoid cyclases should have evolved from a common ancestor cyclase; that is, new catalytic sites have evolved in different biological species by introducing the specific amino acid residues into an ancestor cyclase. The cyclization reaction proceeds through the generation of a series of discrete carbocation intermediates; Johnson[5] has proposed the idea that negative point charges involved in the enzyme would be responsible for the cyclization reaction with regio- and stereochemical control by stabilization of the specific carbocation transition. There have been remarkable advances during the last five to six years in the gene cloning of SHCs and OSCs from many different biological species.[6-21] The deduced amino acid alignments have revealed that the aromatic amino acids are unusually abundant; therefore, some workers have hypothesized that the cyclization reaction would be accelerated by cation/π-interaction between the aromatic residues and the cationic transition formed during the cyclization of **1**.[11,14,22-24] The specific amino acid repeats [(K/R)(G/A)X_{2-3}(F/Y/W)(L/I/V)X_3QX_{2-5}GXW], the so-called QW motifs,[22] have been found in all the families of prokaryotic SHCs and eukaryotic OSCs, and the aromatic amino acids, specifically with tryptophan due to its high π-electron density, of the QW motifs have been presumed to correspond to the negative point charges.[14,22-24] One or two QW motif(s) have also been found for other terpene synthases such as *ent*-kaurene[25] and abietadiene cyclases.[26] This hypothesis for the QW motifs seems to be acceptable, but no experimental proof has been presented.

An X-ray analysis of SHC from the thermoacidophilic bacterium, *Alicyclobacillus acidocaldarius*, has recently been reported, revealing that the repetitive amino acids of all the QW motifs are incorporated into two kinds of secondary structure, *i.e.*, α-helices and the stacking structure of glutamine with tryptophan, and are located near the surface of the dumbbell-shaped protein.[27] Therefore, it is unlikely that the QW motifs interact with the substrate. Wendt *et al.*[27] have postulated that the QW motifs may work to stabilize or reinforce the protein structure, but no definitive evidence has been given. The point mutation of QW motif 5a* has resulted in complete loss of the enzyme activity, leading us to propose in the previous paper that the tryptophan of QW motif 5a is an active site.[28] This result drawn from muta-

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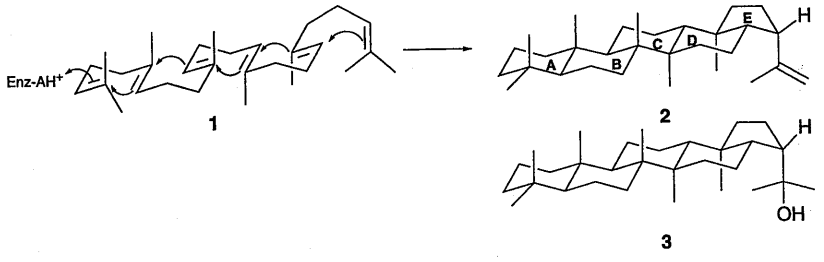
Abbreviations: SHC, squalene-hopene cyclase; OSC, oxidosqualene cyclase

* The numbering of the eight QW motifs is according to ref. 20.



Scheme 1. Cyclization Mechanism for Squalene **1** into Hopene **2** and Hopanol **3**.

;enesis experiments is inconsistent with the conclusion nferred from an X-ray analysis that all the QW motifs eside outside the enzyme cavity.[27] In order to resolve his inconsistency and end the historical dispute about he function of the QW motif, we carried out site-direct-d mutagenesis experiments, by which all the conserved ryptophans were replaced by phenylalanine or aliphatic mino acids.

To date, little has been reported on the enzyme kinet-cs of the mutated SHC to clarify the tryptophan func-ion of the QW motifs; therefore, their functions have emained inconclusive. Our site-directed mutagenesis ex-periments on SHC from *Alicyclobacillus acidocaldarius* ave revealed that mutations of QW motifs 4* and 5a* narkedly lowered the optimal temperature and the legree of catalytic activity, while mutations of the other notifs did not have any marked effect. We report here hat motifs 4 and 5a among the eight motifs greatly con-ribute to stabilizing the enzyme structure against ther-nal denaturation. The other conserved tryptophans esiding outside the QW motifs were also subjected to *in itro* mutagenesis, leading to the indication that these ryptophan residues also work for stabilizing the protein tructure. Only three tryptophan moieties (W169, W312 nd W489) among all the 15 tryptophans conserved in ll the SHCs are proved to play a critical role in the cata-ytic activity. SHC also catalyzes 3(*R*, *S*)-2,3-oxidosqua-ene to produce hydroxylated hopene derivatives. The ole of QW motifs in the cyclization reaction of 2,3-ox-dosqualene is also reported.

Materials and Methods

Site-directed mutagenesis. All site-directed mutagene-is experiments were performed by using the Unique Site limination kit (Pharmacia). Four pUC119 derivatives aving a short fragment of the *shc* gene (pX, pKS, pSB nd pBH) were prepared as the templates for the muta-;enesis reactions. pX, pKS, pSB and pBH designate ach fragment of 910-bp *Xba* I, 1-kbp *Kpn* I-*Sac* I, 396-p *Sac* I-*Bam*H I and 755-bp *Bam*H I-*Hind* III sites, espectively. The pX template was used for the mutation f W32, pKS for the mutations of W78, W142, W169, W258, W312, W339, Q344, W351, W406 and Q411, SB for the mutation of W417, W485 and W489, and BH for the mutation of W558 and W591. As the selec-ion primer characteristic of the Unique Site Elimina-ion protocol, 5′-pd[GACTTGGTTGA**CGCGT**CAC-AGTCA CAG]-3′ was designed to eliminate *Sca* I site n pUC119.

For the mutageneses, the following primers were used.
W32V: 5′-pd[CAGAAG**GGGCCCCCA*CAC***GTAGCC-TTC]-3′ (*Apa* I)
W78L: 5′-pd[GAGGACGGCACG*TTG*GCCCTGTAC]-3′ (*Apa* I)
W169V: 5′-pd[GAGTTTGGCTCG***GTG***GCCCGGGC-GAC]-3′ (*Sma* I)
W169F: 5′-pd[GAGTTTGGCTCG*TTC*GCCCGGGCG-AC]-3′ (*Sma* I)
W169H: 5′-pd[CGAGTTTGGCTCG*CAC*GCCCGGG-CGACCG]-3′(*Sma* I)
W258L: 5′-pd[CGGAGACGG**A**AGC*TTG*GGCGGGA-TTC]-3′ (*Hind* III)
W339L: 5′-pd[CGCTTGGTCAAGGCCGGCGAG*TT-G*CTGTTGGACCGG]-3′ (*Nae* I)
Q344G: 5′-pd[GGCTGTTGGACCGG***GGG***ATCACG-GTCCCGGGCGAC]-3′ (*Sma* I)
W406V: 5′-pd[GGATTCCGC***GTG***ATTGTCGG**A**AT-GCAG]-3′ (*Sph* I)
Q411G: 5′-pd[GATTGTCGGCATG***GGG***AGCTCGA-ATTC]-3′ (*Sph* I)
W417A: 5′-pd[GTCGTAGGCGCC***GGC***ACCGCCGT-TCG]-3′ (*Nae* I)
W417F: 5′-pd[GTCAACGTCGTAGGCGCC***GAA***AC-CGCC]-3′ (*Sal* I)
W485V: 5′-pd[CCCCAACGACCGAA*CAC*GCTGCC-GTC]-3′ (*Pvu* II)
W489L: 5′-pd[GTAATTGACGCC*CAG*TCGACCGA-ACCAG]-3′ (*Sal* I)
W489F: 5′-pd[GACGCC***GAA***ACGACCGAACCAAC-TGCCG]-3′ (*Pvu* II)
W522V: 5′-pd[CTGATGCTGCTCGAC***GAC***GTCGA-GCGC]-3′ (*Aat* II)
W533A: 5′-pd[CGGCAGTCCTCGCC***GGC***GCCGCC-GTC]-3′ (*Nae* I)
W558L:5′-pd[CGCCATCAGCGC*TAGC*GCCGTCTG-CGAC]-3′ (*Nhe* I)
W591L: 5′-pd[CGGTGTAGTACGGCTCATC*TAGA*C-CGCCGTCCGGG]-3′(*Xba* I)

The bold letters designate the altered bases, and the target mutations are shown in italics. The underlined letters show the silent mutation for easily screening the desired mutants by a restriction fragment analysis, and the created or deleted restriction sites are shown in parentheses. The double-stranded pX, pKS, pSB and pBH plasmids were first denatured by heating (98°C for 7 min). The selection primer and each target mutagenic primer were simultaneously annealed to the same strand of the circular single-stranded plasmid DNA (55°C for

5 min and then 4°C for 10 min).New double-stranded DNA was synthesized by reactions using T4 DNA polymerase and T4 DNA ligase (37°C for 1 hr and then 85°C for 15 min). The mutated plasmid DNA was treated with *Sca* I. The desired DNA could be easily selected from the digested mixture by transforming into a repair-defective (mut*S*) strain of *E. coli* NM522, because the transformation efficiency of non-mutated DNA is poor due to the linearization upon digestion. Plasmid DNA isolated from the transformed cells was subjected to a second round of *Sca* I selection to increase the proportion of the mutant plasmid. After repetitive selection with *Sca* I, the digested plasmid mixture was finally transformed into *E. coli* JM109. Plasmid DNA having the target mutation was further screened by each restriction fragment analysis of the transformed colonies. To confirm that the desired mutation had taken place, the entire region of the inserted DNA was sequenced. Replacement of each fragments (*Nde* I-*Kpn* I, *Kpn* I-*Sac* I, *Sac* I-*Bam*H I or *Bam*H I-*Hind* III) of the wild-type *shc* gene with those of each mutated DNA fragment enabled the pETSHCs mutant to be obtained, this being further confirmed by a restriction fragment analysis. The pETSHCs mutant of W142L, W142F, W312L, W312F, W351L, and W351F have been obtained as described in the previous paper.[28]

DNA sequence analysis. DNA sequencing was carried out by using Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham). Automated sequencing was performed with a LIC-4000 (LI-COR) instrument.

Protein purification. The purification procedure has been described in the previous report,[28] although the heat treatment at 60°C for 10 min was not performed to prevent possible thermal denaturation of the mutated enzyme, despite heating being a good protocol to denature the *E. coli* protein due to the thermophilic nature of wild-type SHC.

Thermal stability and kinetic analysis. The reaction mixture contained 60 mM sodium citrate (pH 6.0), 0.5 mM squalene, 0.2% Triton X-100, and 5 μg of purified enzyme in a final volume of 5 ml. Incubation was conducted at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C or 70°C for 1 hr and terminated by adding 6 ml of 15% methanolic KOH. Enzymic products **2** and **3**, and starting material **1** which remained unreacted were extracted four times with 5 ml of hexane, and identified and quantified by GLC in a DB-1 capillary column (30 m) at 270°C. To determine the optimal temperature for the reaction of 0.5 mM 3(*S*)- **4** or 3(*R*)-2,3-oxidosqualene **7**, incubation was performed under the same condition as those for the squalene reaction. GLC was carried out with a slower flow of carrier gas than that for squalene to enable good separation and quantification of products **5, 6, 8** and **9**. Each incubation was carried out at 30°C for 1 hr; no thermal denaturation of the enzyme being apparent; kinetic parameters K_m and V_{max} were then analyzed from Lineweaver-Burk plots. All SHCs, examined, including the wild-type and the mutants were converted to **2** and **3** from **1** in a ratio of ca. 5:1, while the ratio of **5** (**8**) to **6** (**9**) from **4** and **7** was nearly equal.

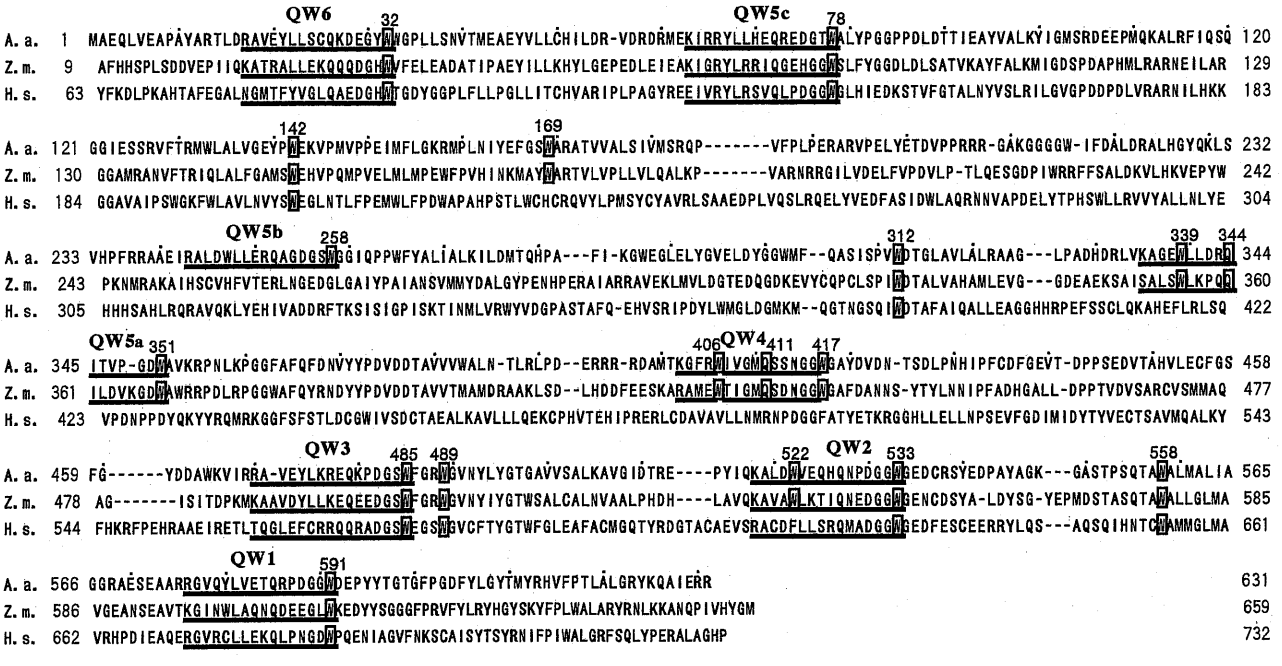


Fig. 1. Amino Acid Alignments of the Cyclases from *Alicyclobacillus acidocaldarius* (A.a., Gram-positive), *Zymomonas mobilis* (Z.m., Gram-negative) and Homo Sapiens (H. s.).

The cyclases of A.a. and Z.m. catalyze the reaction of squalene into hopene and hopanol (SHC), while the enzyme of H.s. (OSC) converts 3(*S*)-2,3-oxidosqualene into lanosterol. The target amino acid residues of SHC for the site-directed mutagenesis are boxed. Lines show the QW motifs.

Results and Discussion

Seven SHCs[6,12,18,20,28] [EMBL Acc. No. X89854, Y09979] and nine OSCs, including lanosterol,[7,8,10,11,13-17] cycloartenol,[9,19] lupeol [Genbank Acc. No. U49919] and β-amyrin synthases,[21] have so far been cloned and sequenced. The number of QW motifs involved in a bacterial SHC depends on the strain: eight motifs for Gram-positive, but seven motifs for Gram-negative bacteria. Eucaryotes have five motifs which are commonly found in both SHCs and OSCs. Fig. 1 depicts the amino acid alignment of two SHCs from *Alicyclobacillus acidocaldarius* (Gram-positive) and *Zymomonas mobilis* (Gram-negative), and of OSC from *Homo sapiens* as typical examples. Three clones of Gram-negative bacteria (*Alicyclobacillus acidocaldarius, Alicyclobacillus acidoterrestris, Synechocystis* sp.) have been sequenced, while four clones of Gram-positive species (*Zymomonas mobilis, Bradyrhizobium japonicum, Rhodopseudomonas palustris*, and *Methylococcus capsulatus**) have been sequenced. All the cyclases of SHCs and OSCs have five common QW motifs [QW 1, 2, 3, 5c and 6, the identity of the QW motif being according to the literature.[20]]. In addition to the five motifs, two other motifs of 4 and 5a supplement the Gram-negative SHCs, and the Gram-positive ones have the further additive of QW motif 5b (Fig. 1). *In-vitro* mutagenesis experiments were targeted on 15 tryptophans, which are completely conserved among all the SHCs, and one tryptophan (W258, QW 5b), which is conserved in the Gram-positive SHCs. Eleven tryptophan residues are located inside QW motifs at position 32, 78, 258, 339, 351, 406, 417, 485, 522, 533 and 591. The five tryptophan residues located outside the QW motifs can be found at 142, 169, 312, 489 and 558. These tryptophan residues are classified into three categories, in respect of their secondary structures. First, the six amino acids of W169, W312, W339, W406, W522 and W558 are involved in the α-barrel helices.[27] Second, the eight tryptophan moieties inside the QW motifs, located at position 32, 78, 258, 351, 417, 485, 533 and 591, are included in the specific secondary structures stacked with the glutamine residue.[27] The W339, W406 and W522 residues residing inside the QW motifs constitute the α-helices, but they do not share the stacking structure with glutamine residue. Third, the other two tryptophans (142 and 489) are not included in any type of the secondary structure for α-helices, β-strands or the stacking formation.[27] To evaluate the function of tryptophan, all the tryptophans were mutated into other amino acids of aromatic phenylalanine or aliphatic valine, leucine and alanine.

Mutation of all the Conserved Tryptophans and Glutamine inside the QW motifs

Figure 2 shows the relationship between the incubation temperature and enzyme activity of the mutants with alteration to the QW motifs 1, 2, 3, 5b, 5c and 6. Wild-type SHC is known to have a catalytic optimum at 60°C and at pH 6.0.[6,28] The mutants of W32V, W78L, W522V, W533A, W485V and W591L had the same activities and optimal temperature as wild-type SHC, but the mutant of W258L (QW 5b) did not, exhibiting decreased activity (60% of the wild type) and a lower optimal temperature (55°C). The lower optimal temperature suggests that thermal denaturation would gradually occur during the progress of the enzymic reaction; thus, the enzyme activity still remained at lower temperatures, but was completely lost at higher temperatures. This fact indicates that this tryptophan residue may work to reinforce the protein structure. The optimal pH values of all the mutants were the same as that of wild-type SHC (data not shown).

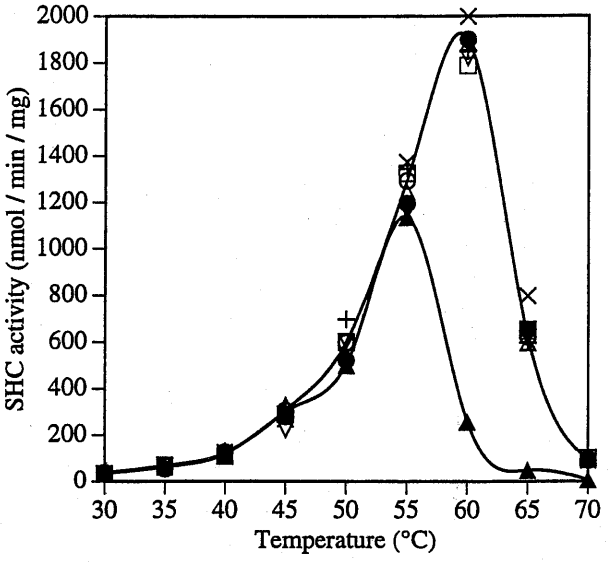


Fig. 2. Enzyme Activities of Mutants Targeted for QW Motifs 1, 2, 3, 5b, 5c and 6 Plotted against the Incubation Temperature.
○, wild-type; ×, W591L (QW motif 1); +, W533A (QW 2); □, W522V (QW 2, α-helix); ▽, W485V (QW 3); ▲, W258L (QW 5b); △, W78L (QW 5c); ●, W32V (QW 6). All the tryptophan moieties are responsible for the stacked structure with the glutamine residue, except for W522 which is involved in the α-helix structure.[27]

Figure 3 shows the enzyme activities of the mutants targeted for the motif 4 against the incubation temperature. The two conserved tryptophans (406 and 417) were changed into aliphatic valine and alanine, and into aromatic phenylalanine in order to identify the function of the indole ring. The mutants of W406V and W417A markedly decreased the enzyme activities in addition to the lower optimal temperatures, being 19% of the wild-type SHC at the optimal temperature of 50°C, and 15% activity at 47°C, respectively. It should be noted that, at 60°C, corresponding to the optimal temperature of wild-type SHC, these two mutants (W406V and W417A) had no enzyme activity. However, when tryptophan was replaced by phenylalanine (W417F), the enzyme activity recovered to 44% of that of the wild-type SHC, in other words; it was *ca.* 3-fold higher than that of W417A, and the optimal temperature was elevated from 47°C to 55°

* In the case of *M. capsulatus* SHC, the three conserved tryptophans described here are displaced by other aromatic amino acids as follows: W78 (QW5c)→Y, W417 (QW4)→F, and W558 (outside QW motif)→F.[20]

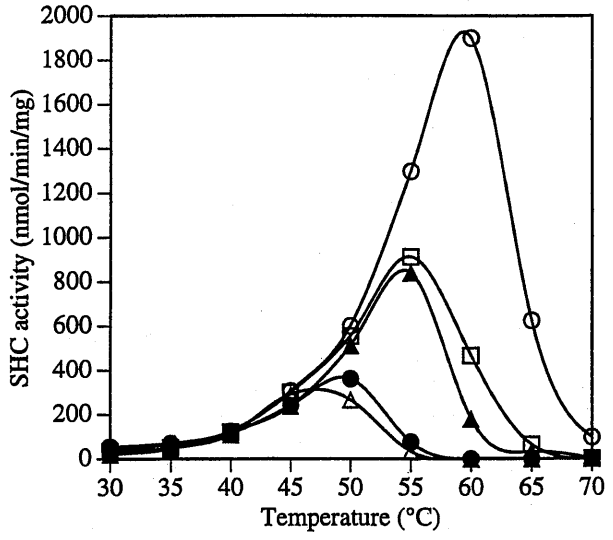


Fig. 3. Relationship between the Cyclase Activities of the Mutants Targeted for QW Motif 4 and the Incubation Temperatures.
○, wild-type; ●, W406V; △, W417A; ▲, W417F; □, Q411G. The W406 residue is composed of part of an a-helix, while the others are part of the QW stacked structure.[27]

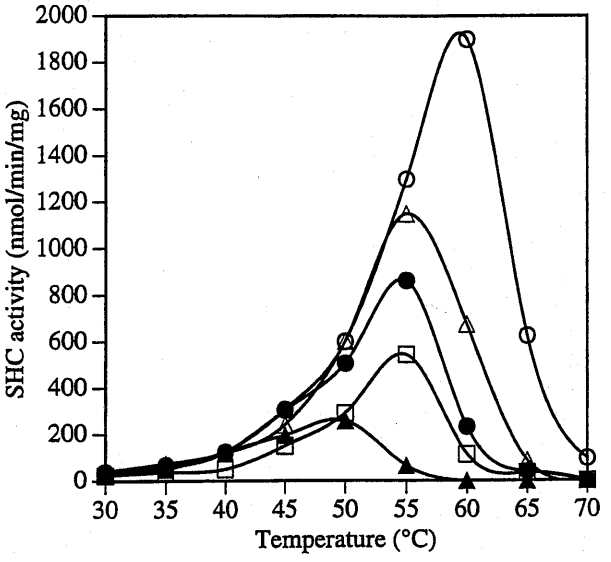


Fig. 4. Dependency of the Incubation Temperature of the mutants Targeted for the QW 5a motif on the Enzyme Activity.
○, wild-type; △, W339L; ▲, W351L; ●, W351F; □, Q344G. The side chain of W351 participates in the stacked structure, but that of W 339 is not responsible for stacking. The kinetic values of W351L and W351F at the given temperature of 60°C have been previously reported.[28]

C. The catalytic activity and optimal temperature decreased in the following order: tryptophan> phenylalanine> aliphatic amino acids, indicating that richness of the π-electron density in the aromatic rings was important for catalysis; the higher the electron density of π-electrons, the greater was the stability of the enzyme structure. The mutation of Q411G was then performed to identify the function of glutamine, the side chain of which has been reported to be stacked with that of tryptophan.[27] If glutamine was to be replaced by glycine, no stacking interaction with tryptophan would be anticipated, because glycine lacks the side chain. The mutation of Q411G also led to lower enzyme activity (48%) and optimal temperature (55°C). This would have been due to the lack of stacking interaction in the Q411G mutant. As the driving force for stacking, CH/π-interaction[29,30] might be proposed, although was not clearly mentioned in the X-ray analysis,[27] this interaction having previously been proposed for substrate binding.[28] Similar findings were also observed with the mutations of QW motif 5a (Fig. 4). A comparison of Fig. 2 with Figs. 3 and 4 reveals that the two motifs 4 and 5a among all eight QW motifs had the most significant role in reinforcing the protein structure against thermal denaturation. Figs. 3 and 4 also show that the catalytic activities of W417A and W351L were completely quenched at 60°C, this being the catalytic optimum for wild-type SHC. This previously led us to propose that the tryptophan residue (W351) of motif 5a was the active site; however, this conclusion was erroneous, because the kinetic data for W351L that have previously been reported were measured only by incubation at 60°C.[28] Figs. 3 and 4 also depict that there was no thermal denaturation at the lower temperature of 30°C, although the enzyme activity was much less than that at the optimal temperature for wild-type SHC (60°C).

Table 1 summarizes the kinetic data for the mutated SHCs that were targeted for all the QW motifs, this being determined by incubating for 60 min at 30°C. All the mutants had almost the same K_m and V_{max} as that of the wild type. This fact strongly demonstrates that not all the QW motifs were the active sites and that the function of the tryptophans that are involved in QW motifs 4, 5a and 5b can be assigned to stabilizing the protein structure, thus indicating that squalene substrate cannot be in contact with all the QW motifs. This idea agrees with the result of the X-ray analysis that all the QW motifs were located near the protein surface and not in the central cavity.[27] However, we cannot discover any function for QW motifs 1, 2, 3, 5c and 6 from the present kinetic investigations, although these five QW motifs are commonly conserved in all the known families of SHCs and OSCs.

Mutations of Conserved Tryptophans Outside the QW Motifs

We have proposed in the previous paper,[28] based on the kinetic results from incubations at 60°C (the optimal temperature for wild-type SHC), that the residues of W169, W312 and W489 are elements of the active sites, because mutation of W169V, W312L and W489L resulted in a complete loss of cyclization activity. Despite changing the incubation temperature (30–70°C), the mutants of W169V and W489L did not exhibit any activity (Figs. 5a and 5b); thus, the residues of W169 and W489 were further proved to be active sites. Replacing W169 and W489 by other aromatic amino acids such as phenylalanine or histidine have enabled the recovery of enzyme activity, as shown in Fig. 5b. Histidine may usually be considered to be a basic amino acid, but the

Table 1. Kinetic Parameters of the Wild-type and Mutant SHCs Targeted for ALL the QW Motifs The kinetic values of K_m and V_{max} were determined from Lineweaver-Burk plots. The enzyme activities were assayed by estimating the amount of hopene produced by incubating at 30°C for 60 min, the purified proteins not being denatured at the temperatures

SHC	Targeted motif	Optimal temp. (°C)	Relative spec. activity at optimal temp.	K_m (μM)	V_{max} (nmol/min/μg)	V_{max}/K_m ($\times 10^3$)	Relative activity (%)
Wild-type	—	60	100	16.7	0.090	5.39	100.0
W32V	QW6	60	100	16.7	0.089	5.33	98.9
W78L	QW5c	60	100	16.3	0.092	5.64	104.6
W258L	QW5b	55	60	17.2	0.091	5.29	98.1
W339L	QW5a*	55	60.5	17.0	0.086	5.06	93.9
Q344G	QW5a	55	28.6	16.5	0.065	3.94	73.1
W351L	QW5a	49	13.7	17.5	0.089	5.09	94.4
W351F	QW5a	55	45.3	17.0	0.090	5.29	98.1
W406V	QW4*	50	19.0	16.8	0.090	5.36	99.4
Q411G	QW4	55	48.0	16.8	0.088	5.24	97.2
W417A	QW4	47	15.8	16.7	0.088	5.27	97.8
W417F	QW4	55	44.2	17.2	0.090	5.23	97.0
W485V	QW3	60	100	16.6	0.090	5.42	100.6
W522V	QW2*	60	100	16.9	0.088	5.21	96.7
W533A	QW2	60	100	16.9	0.094	5.56	103.2
W591L	QW1	60	100	17.3	0.091	5.26	97.6

* This motif consists of part of the outer barrel helix, whereas the others comprise the stacked structure between each side chain of the tryptophan and glutamine residues.

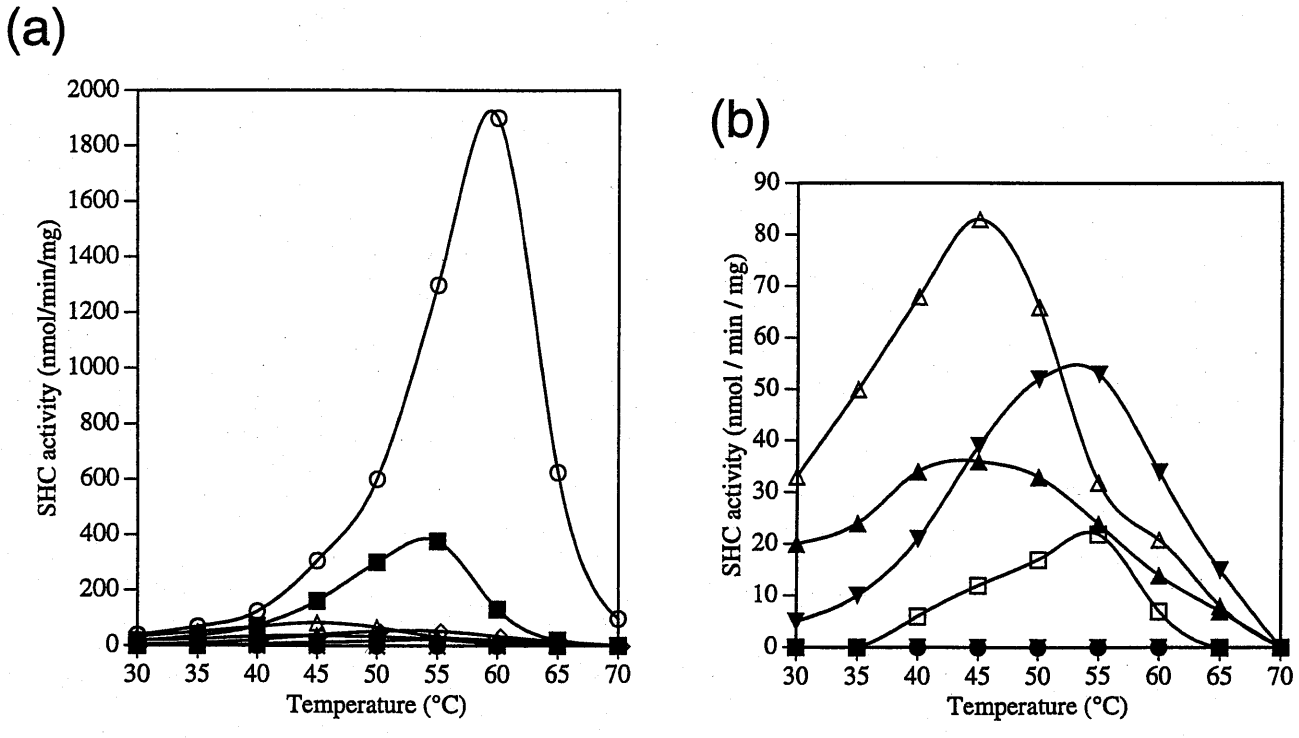


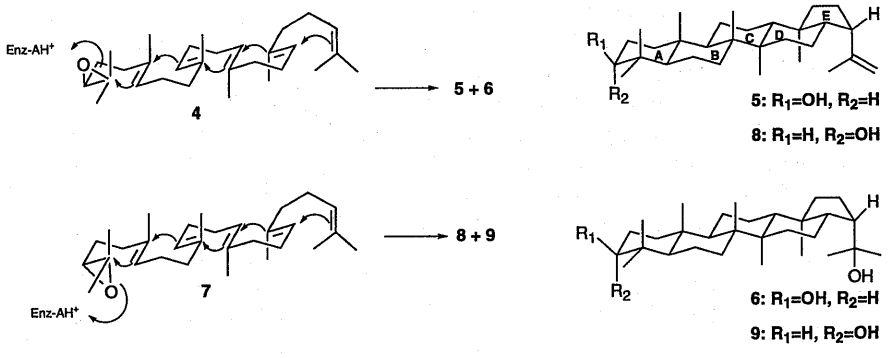
Fig. 5. (a)Enzyme Activities of Active Centers *versus* the Incubation temperature.
○, wild-type; ●, W169V; △, W169F; ▲, W169H; □, W312L; ■, W312F; ▲, W489L; ▼, W489F. (b) An enlarged Part of (a).

π-electron of His234 in lanosterol synthase, *i.e.* of aromatic nature, has been assumed to work for stabilizing the carbocation intermediate produced during cyclization.[31] The isolation of a dammarene compound from the incubation experiments on W169F, W169H and W489F has allowed us to propose a new cyclization mechanism: the 6-membered D-ring of the hopene skeleton is formed through a ring expansion process of the 5-membered D-ring intermediate.[32,33] In addition, it has been supposed that, during the cyclization reaction, W169 may be located near the D-ring of hopene skeleton inside the enzyme cavity.[33] Fig. 5a also shows that W312 was a possible active site, because the activity of the W312L mutant was almost quenched, and the activity of this mutant recovered to 20% of that of the wild type (also see Table 2). The catalytic optimum temperatures for the mutants were lower than that for the wild type (Fig. 5b): 45°C for W169F and W169H, 54°C for

Table 2. Kinetic Parameters of the Wild-type and mutant SHCs The mutations were targeted for the tryptophans located outside QW motifs. The three residues W169, W312 and W489 are components of the active sites. The other two tryptophans W142 and W558 are not active sites. The incubation conditions are the same as those in Table 1.

SHC	K_m(μM)	V_{max} (nmol/min/μg)	V_{max}/K_m ($\times 10^3$)	Relative activity (%)	Optimal Temp. (°C)	Specific activity at optimal temp.
Wild-type	16.7	0.090	5.39	100.0	60	100
W142L	17.9	0.090	5.03	93.3	55	21.1
W142F	17.2	0.089	5.17	95.9	55	28.9
W169V*	—	—	—	0	—	0
W169F	275.6	0.076	0.28	5.2	45	4.4
W169H	280.0	0.033	0.12	2.2	45	2.8
W312L*	—	—	—	0	54	1.2
W312F	55.1	0.082	1.49	27.6	54	19.8
W489L*	—	—	—	0	—	0
W489F	91.9	0.013	0.14	2.6	53	2.9
W558L	16.8	0.090	5.36	99.4	58	68.4

* No activity was detected with a large quantity of the protein (1.5 mg), this amount being 300-fold that of the usual assay (5 μg); therefore, the kinetic values were not determined.



Scheme 2. Conversion of 3(*S*)- **4** and 3(*R*)-2,3-Oxidosqualene **7** into 3β- **5** and 3α-Hydroxyhopene **6**, and 3β- **8** or 3α-Hydroxyhopanol **9** by SHC.

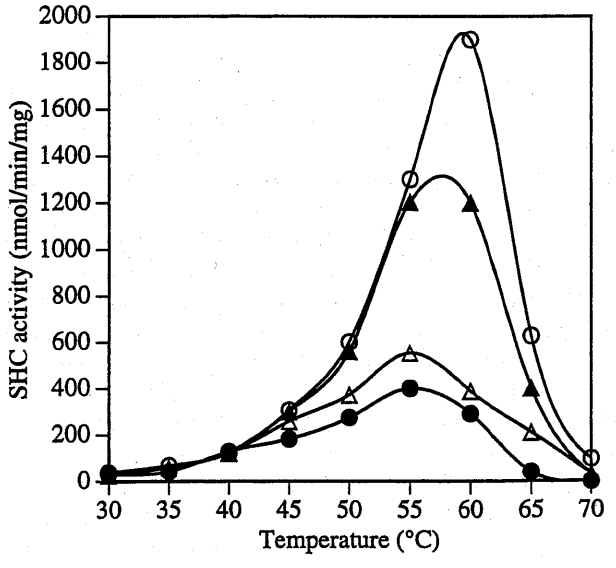


Fig. 6. Enzyme Activities of these mutants not involved in either in the Active Center or the QW Motifs.
○, wild-type; ●, W142L; △, W142F; ▲, W558L. The W558 residue comprises an α-helix.[27]

W312L and W312F, 55°C for W142L and W142F, 53°C for W489F, and 58°C for W558L (Table 2). The optimal pH values of all the mutants were the same as that of the wild type. This finding suggests that mutation of the conserved tryptophans present inside the central cavity also leads to instability of the protein structure in a similar way to the case of surface-located QW motifs 4, 5a and 5b. The kinetic results are summarized in Table 2, which were determined after incubating at pH 6.0 and 30°C and for 60 min. At 30°C, no thermal denaturation was apparent with any of the mutants (Figs. 5 and 6). With the mutants of W142L, W142F and W558L, the K_m and V_{max} were the same as those of the wild type, indicating that W142 and W558 did not have a catalytic function. In contrast, the kinetic values for the mutants of W169F, W169H and W489F were quite different from those of wild-type SHC (Table 2). We have previously suggested for the cyclization mechanism that W169 acts as the binding,[33] possibly *via* a CH/π complex,[28-30] and that W489 has two roles in binding and carbocation stabilization through the cation/π-interaction.[33]

Table 3. Comparison of the Kinetic Data for Oxidosqualenes with a Squalene Substrate for Purified Wild-type SHC (5 μg) Incubated at 60°C for 10 min (R+S)-OS, S-OS, and R-OS stand for a racemic mixture of 3(*R,S*)-oxidosqualenes, 3(*S*)-**4**, and 3(*R*)-2,3-oxidosqualene **7**, respectively. These kinetic values were determined by using the racemic mixture. The kinetics of (R+S)-OS were determined from the total amounts of **5** and **8** produced. The kinetic results of S-OS and R-OS were obtained from the amounts of **5** and **8** produced, respectively. It is known that **5** and **8** are each produced from **4** and **7**, respectively.[38] The numbering of the compounds is shown in Scheme 2.

Substrate	K_m (μM)	V_{max} (nmol/min/μg)	V_{max}/K_m ($\times 10^2$)	Relative activity (%)
Squalene	16.7	4.03	24.1	100
(R+S)-OS	1.74	0.53	30.5	126.6
S-OS	0.84	0.33	39.3	163.1
R-OS	2.63	0.20	7.6	31.5

Cyclization Reaction of Oxidosqualene by SHC

The OSC enzymes catalyze 3*S*-oxidosqualene, but are inert to the 3(*R*)-isomer and squalene.[1] However, SHC enzymes are active to both the 3(*R*)- and 3(*S*)-2,3-oxidosqualene enantiomers to give the following products: 3β-hydroxyhopene **5** and 3β-hydroxyhopanol **6** from 3(*S*-)-2,3-oxidosqualene **4**, and 3α-hydroxyhopene **8** and 3α-hydroxyhopanol **9** from 3(*R*)-2,3-oxidosqualene **7** (Scheme 2).[34-38] However, no details of the kinetic investigations on oxidosqualene substrates with wild-type SHC have yet been reported. Squalene was subjected to the reaction with *N*-bromosuccinimide followed by K_2CO_3, oxidosqualene thus prepared being a racemic mixture of **4** and **7**. A mixture of racemates **4** and **7** was incubated to evaluate the kinetic data. Figs. 7 and 8 show that the optimal temperature was 64°C for the formation of **5**, but was 61°C for the formation of **8**, these values being slightly higher than that of the squalene substrate. Table 3 shows the kinetic results obtained from the incubation of a racemic mixture of **4** and **7** with wild-type SHC at 60°C. The K_m value (1.74 μM) of the racemic mixture was determined by estimating the sum of **5** and **8** produced and was much smaller than that of squalene (16.7 μM), suggesting stronger binding of the oxidosqualene racemate to SHC. However, in the case of tetrahymanol cyclase, the K_m values of oxidosqualene and squalene have been reported to be nearly equal.[36] The V_{max} value (0.53 nmol/min/μg) of the oxidosqualene racemate was smaller than that of squalene (4.03 nmol/min/μg), indicating a faster reaction with true substrate **1** than that with oxidosqualene racemates **4** and **7**. The reason for this is not clear, but the negative point charges that are responsible for cation stabilization might operate improperly for oxidosqualene substrates in the geometry of the SHC cyclase. Due to the higher affinity of oxidosqualenes than squalene, it was examined whether the cyclization reaction of squalene would be inhibited or not by the oxidosqualene racemates. From Lineweaver-Burk plots, inhibition constant K_i was determined to be 9.46 μM (IC_{50}=32 μM). To compare the kinetic data of 3(*S*)-**4** with those of 3(*R*)-**7**, the formation of products **5** and **8** was separately quantified by GLC. A comparison of each of the enantiomers showed that the 3*S*-isomer (K_m=0.84 μM) bound more strongly to SHC than to the alternative (K_m=2.63 μM). The V_{max} values of the 3*S*- and the 3*R*-isomers at 60°C were 0.33 and 0.20 (nmol/min/μg), respectively, suggesting that the formation of **5** was more kinetically favored (faster) than that of **8**. The relative activity (V_{max}/K_m) of the 3*S*-isomer was highest among the three substrates (Table 3). The existence of this selectivity has never before been reported. We also examined the role of all the QW motifs in the cyclization of oxidosqualene in order to compare with the case of the squalene substrate (Figs. 7 and 8). All mutated SHCs targeted for QW motifs had retained their catalytic activity, indicating that the QW motifs were not also active sites for oxidosqualenes, as was revealed for the squalene substrate. Figs. 7 and 8 were used to determine the following optimal temperatures for the oxidosqualene substrates: for the 3*S*-isomer, W591L (64°C, QW1) ≈ W533A (64°C, QW2) ≈ W485V (64°C, QW3) ≈ W32V (64°C, QW 6) ≈ W78L (64°C, QW5c) > W258L (60°C, QW5b) > W351F (59°C, QW5a) > W417F (57°C, QW4) > W351L (52.5°C, QW5a) > W417A (51.5°C, QW4); for the 3R-isomer, W591L (61°C) ≈ W533A (61°C) ≈ W485V (61°C) ≈ W32V (61°C) ≈ W78L (61°C) > W258L (59°C) > W351F (58°C) > W417F (56.5°C)

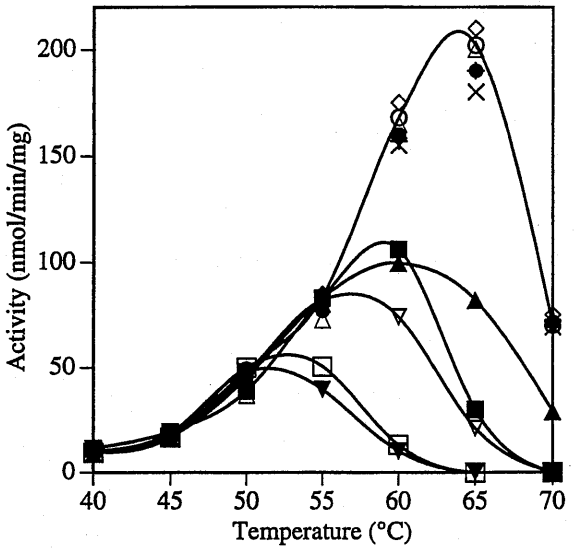


Fig. 7. Enzyme Activities of the 3(*S*)-2,3-Oxidosqualene Substrate with Wild-type SHC and the Mutants Plotted against Incubation Temperature.

Activity was estimated from the produced amount of 3β-hydroxyhopene after incubating for 60 min at pH 6.0 with purified SHC (5 μg). ○, wild-type; ×, W591L (QW 1); +, W533A (QW 2); ◇, W485V (QW 3); ▽, W417F (QW 4); ▼, W417A (QW 4); □, W351L (QW 5a); ■, W351F (QW 5a); ▲, W258L (QW 5b); △, W78L (QW 5c); ●, W32V (QW 6).

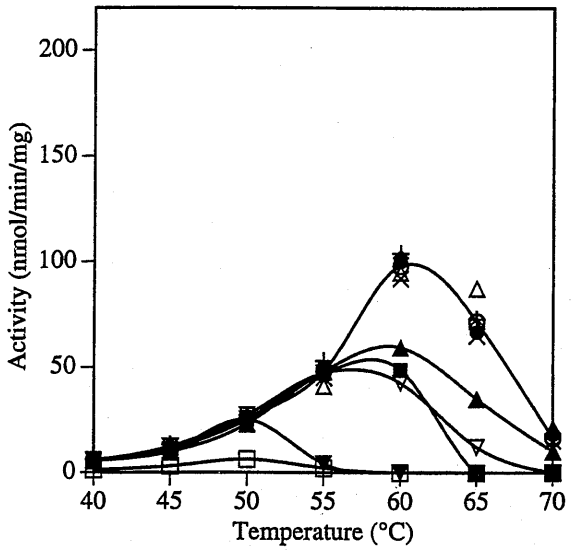


Fig. 8. Formation of 3α-Hydroxyhopene from 3(*R*)-2,3-Oxidosqualene.

The incubation conditions and symbols are the same as those in Fig. 7.

>W351L (50°C)>W417A (50°C). The optimal temperatures for the 3*S*-isomer were higher than those for the 3*R*-isomer. The stability against heat denaturation for the oxidosqualene reaction were similar to that for squalene (compare Figs. 2–8 and Tables 1–3); the role of the two QW motifs 4 and 5a was also true for the oxidosqualene cyclization.

In summary, it is apparent that while QW motifs 4 and 5a played a significant role against thermal denaturation, but they are unlikely to be active sites despite the marked loss of cyclase activity. However, none of the other motifs gave much contribution to heat stability. Wendt *et al.* have estimated the released energy to be *ca.* 200 kJ/mol for the formation of pentacyclic hopene.[27] Corey *et al.* have reported the exothermic energy of each ring closure to be *ca* 83 kJ/mol,[39] for the oxidosqualene cyclization, resulting in a high energy release for complete construction of the tetracyclic lanosterol skeleton. The high energy released during the polyene cyclization reaction would degrade the protein structure, so Wendt *et al.* have proposed an energy absorption function for the QW motifs. However, the present study has validated this assumption just for the three QW motifs 4, 5a and 5b. Thermal instability may arise from degradation of the stacking structure of these QW motifs, because displacement of the tryptophans and glutamines that share the stacking with each other, resulted in a lower optimal temperature. It is worth noting that the mutants replaced by phenylalanine could be more stabilized than those by aliphatic moieties. This result agree with the report that the conservative substitution of phenylalanine for eight tryptophans of lanosterol synthase retained catalytic activity.[17] It is likely that aromatic indole moieties embody the reinforcement of the protein structure. The conserved tryptophan residues that are responsible for active centers are also presumed to have a stabilizing effect on the protein structure, because mutants W169F, W169H and W489F had a catalytic optimum at lower temperatures than wild-type SHC (Fig. 5 and Table 2). The structure of the cyclase protein might be integrated in part by CH/π or π/π interaction of the indole nuclei with the side chains of aliphatic amino acids or with those of other aromatic phenylalanine and tyrosine, respectively. The indole ring is stronger than the phenyl ring for CH/π interaction.[29,30] Thus, the mutants (W→F) would have had less catalytic activity at higher temperatures than wild-type SHC, because of the poor structural integration of the mutated cyclases. Bacterial hopanoids are considered to act as membrane reinforcers comparable to sterols in the membranes of eucaryotes;[20] *i.e.*, they probably play important roles for adaptation to high temperature (*A. acidocaldarius*), to a high concentration of ethanol (*Z. mobilis*), and to detrimental oxygen gas (*B. japonicum*). QW motifs 4, 5a and/or 5b, which are specific to bacterial SHCs, may contribute to protect the cyclase enzymes against such severe circumstances. The function(s) of the other five QW motifs 1, 2, 3, 5c and 6, which are commonly found in all the families of SHCs and OSCs, has not yet been discovered. A question remains as to why the five common QW motifs have been retained or conserved in all the triterpene cyclase families of SHCs and OSCs during process of evolution. Further studies are required, to get better knowledge about the functions of the five common QW motifs.

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