

Functional analysis of phenylalanine 365 in hopene synthase, a conserved amino acid in the families of squalene and oxidosqualene cyclases†

Tsutomu Hoshino* and Tsutomu Sato

Department of Applied Biological Chemistry, Faculty of Agriculture, and Graduate School of Science and Technology, Niigata University, Ikarashi, Niigata 950-2181, Japan. E-mail: hoshitsu@agr.niigata-u.ac.jp

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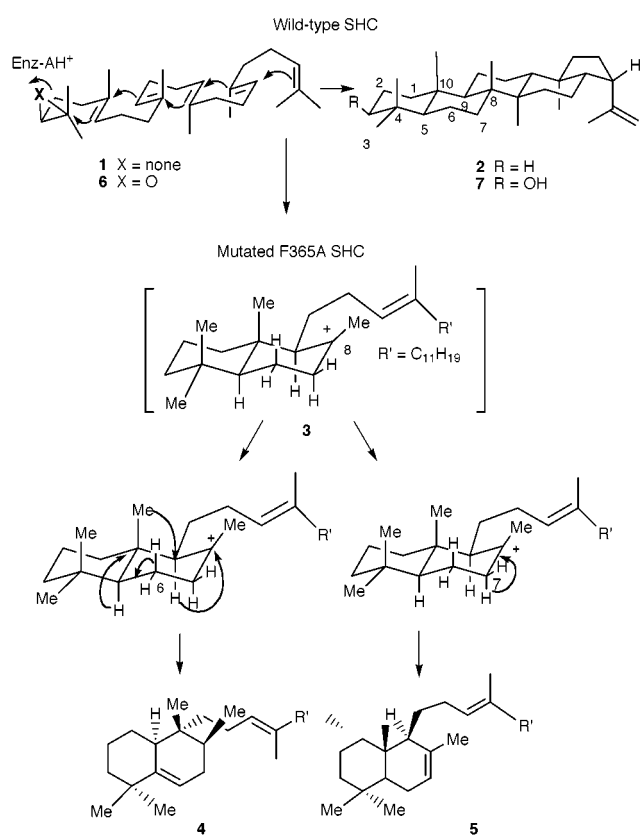
Two bicyclic products were accumulated by the mutant F365A, showing the amino acid residue is located close to the transient C-8 carbocation intermediate in the active site cavity; the mutants of F365Y and F365W significantly accelerated the cyclization reaction at low temperatures.

The cyclization of squalene **1** into the pentacyclic triterpene hopene **2**, mediated by the prokaryotic squalene-hopene cyclase (SHC), is one of the most sophisticated biochemical reactions (Scheme 1).¹ Oxidosqualene also undergoes the polyolefin cyclization by eukaryotic cyclases, in a similar manner to **1**.¹ Recent investigations on the molecular biology of the two cyclases² and on the substrate analogues^{1,3} have encouraged mechanistic studies on the polycyclization reactions. The acyclic molecule **1** is believed to be folded into an all pre-chair conformation inside the enzyme cavity,¹ but the involvement of a ring expansion process has recently been disclosed with respect to the D-ring formation.^{4a,b} Such a ring expansion reaction also occurs in C-ring formation by lanosterol synthase.^{4c,d} An X-ray crystal structure of the SHC from *Alicyclo-*

bacillus acidocaldarius has been reported and a reaction mechanism proposed.⁵ However, site-directed mutagenesis experiments are required to verify the catalytic functions of the residues of the presumed active center. To date, only a few essential components of these active sites have been demonstrated by point mutation experiments.^{4b,6} The polycyclization cascade has been proposed to proceed by the stabilization of discrete carbocation intermediates *via* cation- π interaction with aromatic amino acid residues of the cyclases,⁷ but no kinetic evidence has been reported. In a series of point mutation experiments conducted by us, Phe365, which is conserved among all the known prokaryotic SHCs and eukaryotic cyclases,^{2,5,6c} has been identified as having a stabilizing function for the transient C-8 carbocation intermediate. We show here kinetic data of the mutated SHCs of F365Y and F365W.

Cell-free homogenates of the *E. coli* clone encoding the mutated F365A SHC, prepared from 8 L culture, were incubated with **1** (105 mg) at pH 6.0 and 55 °C for 16 h. The GC analysis showed the presence of two major products (**4** and **5**), a little of **2** and recovered **1** in the reaction mixture. Separation on a SiO₂ column using hexane as an eluent afforded **4** (27.8 mg), **5** (25.1 mg), **2** (3.0 mg) and **1** (32.4 mg). Analyses of the 2D NMR spectra revealed that the structures of **4** and **5** had a 6/6-fused bicyclic skeleton.⁸ Formation of **4** and **5** suggested that the sequential cyclization reactions are quenched at the bicyclic stage of the putative intermediate **3**. A series of 1,2-shifts of hydrides and a methyl group in an antiparallel fashion could trigger the deprotonation at C-6 to produce **4**, while a deprotonation at C-7 could give rise to **5** (Scheme 1). These findings indicate that the F365 residue is critical to the completion of the polycyclization and that the π -electron of this side chain may stabilize the transient C-8 carbocation. There were no detectable amounts of mono- or tri-cyclic products, further suggesting that the F365 residue is close to the C-8 cation in the enzyme cavity, but not near the mono- and tri-cyclic intermediary carbocations, which is consistent with the recent suggestion inferred from X-ray analysis.⁵

To validate the proposed role of the cation- π interaction, the mutants F365Y and F365W were constructed based on the idea that the higher electron density of the π -electrons, the faster cyclization reaction. Fig. 1 depicts the specific activities of these mutants against incubation temperatures. The mutant F365Y completed the polycyclization to the final product **2** without any intermediate being observed, differing from F365A, and exhibited a remarkable increase in the reaction rate at low temperatures (10–50 °C); the F365Y produced **2** even at a temperature as low as 10 °C, a temperature at which the wild-type SHC has no significant activity. The activation energy (E_{act}), estimated from the Arrhenius plots,⁹ was greatly reduced; e.g. 37.0 kJ mol⁻¹ for the F365Y compared with 50.1 kJ mol⁻¹ for the wild-type. The higher electron density of the tyrosine residue apparently gives rise to a faster cyclization reaction. The K_m values of the wild-type and F365Y were determined from the Lineweaver-Burk plots to be 16.9 ± 0.6 (30–60 °C) and 502 ± 12 μ M (10–50 °C), respectively, suggesting that the substrate affinity for F365Y was greatly decreased. An unusual profile was found between activities and temperatures (Fig. 1). Three



† Spectroscopic data for **4** and **5**, Arrhenius plots for **1** and **6** and CD spectra for the relevant SHCs are available from the RSC web site, see <http://www.rsc.org/suppdata/cc/1999/2005/>

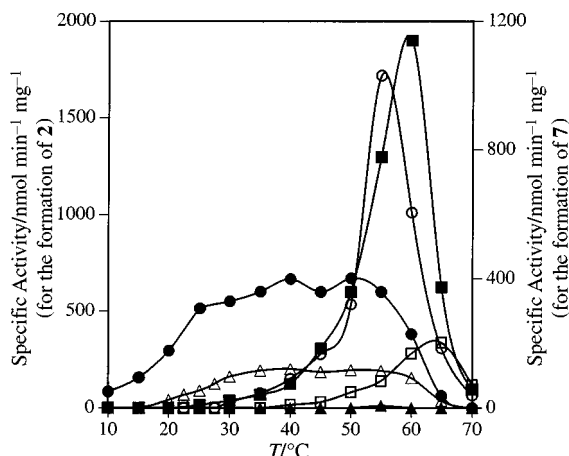


Fig. 1 Specific activities for the formations of **2** (closed symbols) and **7** (open symbols) against incubation temperatures are given for the wild-type SHC (■, □), the mutants of F365Y (●, ○) and F365W (▲, △). One mg of **1** or **6** was incubated with 5 μ g of the homogeneously purified SHCs for 60 min at pH 6.0.

distinctive phases were observed: at 10–25°C, the activities were increased; at 30–50 °C, a steady state was reached; at 55–70 °C, the activities decreased due to irreversible thermal denaturation.¹⁰ The enzyme activity of the F365W was negligible (Fig. 1) despite the higher electron density of the indole ring, which may result from the significantly decreased binding of **1** to the catalytic site. The CD spectra of the three mutants were superimposable on that of the wild-type. However, significant local change may be indeed likely and this may have no visible effect on the overall CD spectra.

(3S)-2,3-Oxidosqualene **6** also undergoes the cyclization by SHC to form 3 β -hydroxyhopene **7**.^{3b,6c,11} Compound **6** was incubated as another enzymic test to obtain further evidence for the cation– π interaction. F365Y had a remarkably enhanced activity, 5-fold higher than the wild-type, and showed a bell-shaped curve (Fig. 1).¹⁰ As expected, F365W also had higher activity than the wild-type at temperatures below 50 °C, but exhibited an unusual profile analogous to that of **1** with the F365Y.¹⁰ No intermediate products were detected in the reaction mixtures of **6** by either F365Y and F365W, whereas the 3-hydroxy derivatives corresponding to bicyclic intermediates were accumulated by the F365A without completion of the polycyclization, further supporting the idea that the residue at 365 must be an aromatic amino acid. The values of E_{act} ⁹ with **6** were 53.6, 49.9 and 42.8 kJ mol⁻¹, respectively, for the wild-type, the F365Y and the F365W mutants, which is in good agreement with the cation– π interaction concept. The enhanced K_m values for these mutants were also observed with **6**; the K_m of the F365W was largest.¹¹

The faster cyclization reactions at lower temperatures with the Tyr and the Trp mutants suggest that the cation– π interaction is likely to occur for the squalene cyclization cascade. The bulky aromatic substituents make the active site region less compact. A somewhat loosely packed protein structure may be more flexible at low temperatures leading to high catalytic activity, as is found for psychrophilic enzymes.^{12a} More detailed studies are required to gain greater insight into the kinetics of these mutants.¹⁰

The previously unknown **4** and the known products **5** (γ -polypodatetraene),¹³ which have a 6/6-fused bicyclic skeleton, were produced by the site-directed mutant of F365A due to the lack of π -electrons, suggesting that the major function of Phe365 would be assigned for stabilization of the C-8 carbocation intermediate possibly *via* a cation– π interaction.

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- Analyses of NMR data (H-H COSY 45, HOHAHA, NOESY, DEPT, HMQC and HMBC) unequivocally supported the structures proposed for **4** and **5**.
- The temperatures for the Arrhenius plots for **1** were in the range of 30–60 and 10–25 °C for the wild-type and for the F365Y, respectively, while in the case of **6** they were 40–60, 30–50 and 20–30 °C, respectively, for the wild-type, the F365Y and the F365W.
- Our working hypothesis for the interpretation of this unusual behaviour is as follows. Reversible denaturation^{12b} may gradually occur from 30 to 50 °C, and the denaturation may be attributable to the more enhanced susceptibility of the mutants to the exothermic high energy, which is released by the cyclization reaction,⁵ because the geometrical change occurred at the active site region (significantly increased K_m). As for the F365Y, temperature dependency for the reaction is different between **1** and **6**. This may be closely related to the increased degree of K_m , leading to the unusual profile for **1** ($K_m = 502 \mu\text{M}$, more sensitive to higher temperatures), but to a bell-shape for **6** ($K_m = 182 \mu\text{M}$, less sensitive). If the denaturation could not occur, the k_{cat} of **1** may have been significantly increased at 30–50 °C. This idea may also be true for the case of **6** by the F365W ($K_m = 357 \mu\text{M}$).¹¹ The denaturation process was confirmed to be reversible; the F365Y was exposed at 40 °C for 60 min and then incubated with **1** at 20 °C, but showed the same activity as that without such treatment. To validate our hypothesis, further evidence is required.
- Kinetic data for the reactions of **6**, measured at 40 °C and for 60 min, are as follows: k_{cat} 1.4, 12.2 and 17.2 min⁻¹, and K_m 0.72, 181.8 and 357.2 μM , respectively, for the wild-type, F365Y and F365W. The more bulky the substituents, the larger the values of K_m . The k_{cat} s and K_m s of the wild-type for the reactions of **1** and **6** at 60 °C are as follows: 288.5 min⁻¹ and 16.7 μM for **1**; 23.6 min⁻¹ and 0.84 μM for **6**.^{6c}
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