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Specific production of γ-polypodatetraene or 17-isodammara-20(21),24-diene by squalene–hopene cyclase mutant

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Abstract—Amino acids lining the catalytic cavity of squalene–hopene cyclase of *Alicyclobacillus acidocaldarius* were mutated to investigate their catalytic functions. Mutagenesis of Leu607 to Lys in the central part of the cavity resulted in the production of the bicyclic γ-polypodatetraene (**1**) as main product, while the mutation of Phe605 to Lys near the deprotonation site of the cavity led mainly to the formation of tetracyclic 17-isodammara-20(21),24-diene (**2**). © 2001 Elsevier Science Ltd. All rights reserved.

In nature a high diversity of cyclic triterpenes does occur.[1] A significant part thereof are products of squalene or oxidosqualene cyclase reactions. The best known triterpene cyclase is squalene–hopene cyclase (=SHC) from the thermophilic bacterium *Alicyclobacillus acidocaldarius*. The X-ray structure from this enzyme has recently been established.[2–4] The SHC is membrane-bound by a hydrophobic area which submerges into the membrane. From this area a channel structure leads to the catalytic cavity, allowing for the entrance of squalene into the enzyme which thereafter folds squalene into a specific pre-reaction conformation. The cavity may be divided into three functional areas: an acidic area for the initiation of the reaction, a mainly hydrophobic area for propagation of the reaction by carbocation stabilization, and a more or less polar area for quenching of the final carbocation by deprotonation. Besides hopene (hop-22(29)-ene= diploptene), the SHC also forms diplopterol (hopan-22-ol) and minor amounts of penta- and tetracyclic products also comprising 17-isodammara-20(21),24-diene (**2**).[5]

Recently, a number of directed mutagenesis experiments have been performed.[6–10] These experiments assigned roles to certain amino acid residues in the catalytic cavity. Asp376 is most probably the protonating group which starts the cyclization cascade. Aromatic residues form the tapestry of the cavity by introducing contours and their π-electrons stabilize

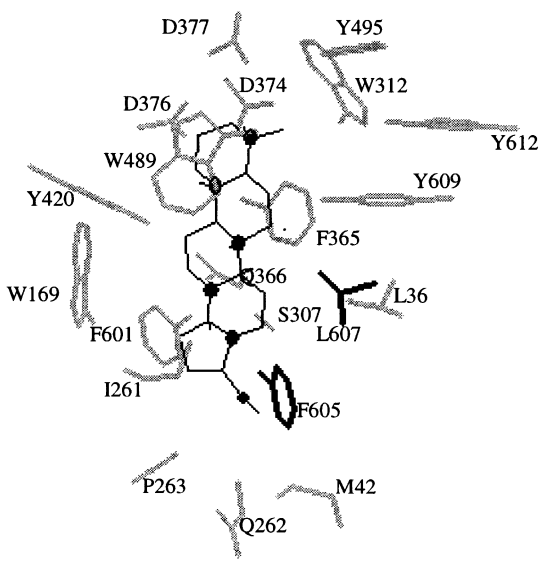

Figure 1. Residues lining the catalytic cavity of SHC in a distance of 6 Å from the modelled-in hop-22(29)-ene molecule. Intermediate carbocations (C4, C10, C8, C13, C17 and C22; starting from the top) in hopene are indicated as black dots (●). F(=Phe)605 and L(=Leu)607 are drawn in black.

Keywords: triterpene synthase; squalene–hopene cyclase; γ-polypodatetraene; 17-isodammara-20(21),24-diene.

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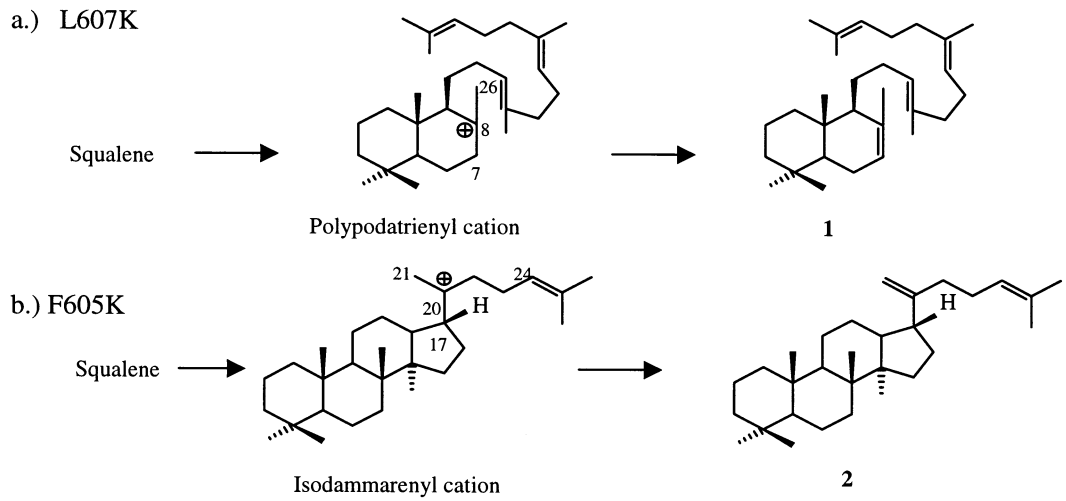

Figure 2. Squalene cyclization to (a) γ-polypodatetraene (**1**) by mutant SHC Leu607Lys and (b) to 17-isodammara-20(21),24-diene (**2**) by mutant SHC Phe605Lys.

intermediary carbocations. Furthermore, the free electron pairs at the OH-group of Tyr420, 609 and 612 may stabilize the intermediate carbocations. Yet, to date all mutagenesis experiments resulted in the simple enhancement of unspecific product formation: hopene is always the main product besides varying amounts of di- to tetracyclic side-products.[6–10]

In contrast, in this paper we describe mutant SHCs which induce a premature termination of the polycyclization reaction leading to bicyclic γ-polypodatetraene (**1**) or tetracyclic 17-isodammara-20(21),24-diene (**2**), respectively as the main products.[11]

Leu607 is located in the central part of the catalytic cavity of SHC pointing with the methyl groups to ring B and C of the modelled-in hopene skeleton (Fig. 1).[3] By substituting Leu by Lys at this position a premature quenching of the intermediate carbocations (bicyclic or tricyclic) was expected.[3] The catalysis by the mutant SHC Leu607Lys is 200 times slower as compared to the wild type.

The GC–MS spectrum of the main product of mutant SHC Leu607Lys was identical to that of γ-polypodatetraene which is found in ferns (**1** in Fig. 2a).[8,14,15] The NMR-data of the isolated product (purity 96% according to GC measurements) are in accordance with the structure of γ-polypodatetraene (**1**) and also with published data.[13–15] The γ-polypodatetraene made up 80% of the products. Additionally, traces of hopene and of other not yet identified components were detected.[12]

The presented data allow a conclusion for the function of Lys607. In the modelled structure of the mutant SHC Lys607 comes approximately 3 Å close to the tertiary carbocation of the bicyclic intermediate (personal communication by Tanja Schulz, Institut für Technische Biochemie, Universität Stuttgart). Assuming that during catalysis Lys is not protonated, this distance between Lys and the tertiary carbocation of the bicylic intermediate should be sufficient for deprotonation. A reason for the low reaction velocity seems to be the fact that the water bound to the γ-amino group of Lys607 will impede the intruding squalene. Hence, our results together with modelling show that the Leu607 in wild type SHC is located near the bicyclic carbocationic intermediate and may have an important steric contribution to chaperone the appropriate conformation of squalene during hopene formation.

The second mutant SHC, namely Phe605Lys, produced 90% 17-isodammara-20(21),24-diene (**2**) (Fig. 2b) besides 10% hopene; the velocity of the reaction was again 200 times lower than that of the wild type. The identity of **2** was demonstrated by the retention time in GC and by the GC–MS spectrum which are both identical to published values.[5] The product spectrum of Phe605Lys is in contrast to SHC mutants Tyr609Phe and Phe365Ala: These mutants form **2** concomitantly with other products.[9,10] The 17-isodammara-20(21),24-diene is a particularly interesting product because it has yet not been found to occur in nature.[1]

It has been proposed that Phe605 stabilizes cation C17, which facilitates the ring expansion to the final six-membered ring D.[3] In mutant SHC Phe605Lys this stabilization is missing. Therefore, the polycyclization stops prematurely. Lys605 may also induce a specific deprotonation at C21 leading to **2**. On the other hand, mutant SHC Phe605Ala led to an unspecific production of ten tri- to pentacyclic skeletons including **2**.[16] This indicates that in this mutant the conformational control and deprotonation specificity are severely disturbed.

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11. *Mutagenesis*. Mutagenesis to SHC Leu607Lys was carried out by a modified 'megaprimer'-method.[17,18] To avoid accidental mutations the 770 bp *Eco*RI-*Xba*I 5′-terminal fragment of the *shc* gene was inserted in pUC18. The mutations were inserted by PCR with a mutagenesis primer and a universal primer outside of the fragment (M13-uni). The megaprimer was obtained in a second PCR reaction together with a second universal primer (M13-rev) and the plasmid. The product was inserted in the *shc* gene by replacing the original fragment. Mutagenesis to SHC Phe605Lys was carried out with QuikChange™ site directed mutagenesis kit from Stratagene.[19]
12. *Extraction*. The enzymatic reaction mixture was extracted with hexane/propan-2-ol (3/2). Triton X100® was removed on a silica gel column (solvent: hexane/ethyl acetate (1/1)). Purification of the hydrocarbons was performed by TLC on silica gel with cyclohexane as the solvent.
13. *NMR-spectroscopy*. The NMR measurements were performed on a Bruker AMX 400 spectrometer. For the structure elucidation of the γ-polypodatetraene (in CD_2Cl_2) ^1H, ^{13}C, DEPT-135, H, HCOSY, HMBC and HSQC experiments were measured at 300 K.
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