Bicyclic triterpenes as new main products of squalene–hopene cyclase by mutation at conserved tyrosine residues

Christine Füll*

Mikrobiologie/Biotechnologie, Universität Tübingen, Tubingen, Germany.

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Abstract The catalytic cavity of the *Alicyclobacillus acidocaldarius* squalene–hopene cyclase is predominantly lined by aromatic amino acids. In mutant cyclases, the four tyrosine residues in the catalytic cavity were replaced by different amino acids. The mutants showed significant differences in catalytic behavior compared to the wild-type and to each other. Mutants Y609L, Y609C and Y609S produced the bicyclic main product γ-polypodatetraene, while Y495L and Y612L showed a wild-type product pattern and produced hopene as the main product. Altered product patterns were also found with Y420 mutations.

Key words: Squalene–hopene cyclase; Triterpene cyclization; Polypodatetraene; Carbocation stabilization; *Alicyclobacillus acidocaldarius*

1. Introduction

The squalene–hopene cyclase (SHC) is found in a large number of bacteria [1]. The *Alicyclobacillus acidocaldarius* SHC was cloned, expressed and mutagenized, and the structure of this monotopic membrane protein was recently solved [2,3]. To date, this SHC is the best characterized triterpene cyclase.

The SHC catalyzes the isomerization of the linear squalene into the pentacyclic hopene (hop-22(29)-ene = diploptene). Hopene is built in a quota of about 95% of the products, while the remaining 5% are mostly diplopterol (hopan-22-ol) but also minor tetracyclic compounds [4].

The complex catalysis occurs in a cavity in the enzyme interior. The hydrocarbon squalene has access to the cavity from the membrane via a channel structure. Within the cavity, squalene is folded and a carbocationic cyclization cascade is initiated by a proton donated from D376, which is one of a trio of aspartates [3,5]. Intermediate carbocations are stabilized by two types of aromatic residues to prevent dead-end products by premature quenching of the reaction. According to the structural arrangement of the amino acids in the cavity (Fig. 1), phenylalanine and tryptophane residues, on the one hand, point with their expanded π-electron systems to the interior of the cavity, and on the other hand, tyrosine residues are oriented with the localized electron pairs of the hydroxyl group towards the cavity to create electron density and an appropriate shape of the catalytic cavity also [3,6].

In nature, about 100 cyclic triterpenes occur as isomers of squalene or oxidosqualene and their structures are compatible with the accepted reaction mechanism of triterpene cyclases [7]. The *A. acidocaldarius* SHC is the model triterpene cyclase to understand this variety. So far, it was possible to create new product patterns for the SHC [8,9], including even those which have not yet been found in nature [10,11]. Alterations of the product pattern have been observed through an exchange of the supposed intermediate carbocation stabilizing residues [6,8,9,11] or through introducing a new proton accepting site [10]. The SHC has also been mutated in the neighborhood of the proton donating D376, thereby shifting the substrate specificity from squalene to oxidosqualene [12]. The resulting successful alteration of the product specificity will be the basis to understand the contribution of specific amino acids during catalysis.

Alteration of product specificity by directed mutagenesis was also achieved with plant triterpene cyclases (for example [13]). A very different approach has been invented by the group of Matsuda, using evolutionary mutagenesis to alter *Arabidopsis thaliana* cycloartenol–oxidosqualene cyclase to a lanosterol producing cyclase (for example [14]).

In this paper we will demonstrate the functional effects of mutations in four highly conserved tyrosine residues in the 'upper' part of the SHC catalytic cavity (Fig. 1). Three of these residues, Y495, Y609 and Y612, are strictly conserved in both SHCs and oxidosqualene cyclases, while Y420 is substituted in some SHCs by phenylalanine, but conserved in the oxidosqualene–sterol cyclases. Our findings show that the effects of mutations on the different tyrosine residues depended on their location in the catalytic cavity and resulted sometimes in a specific alteration of the product pattern.

2. Materials and methods

2.1. Mutagenesis

Mutagenesis was carried out using the QuikChange™ site-directed mutagenesis kit from Stratagene [15]. To avoid additional accidental mutations in *shc* and to lower the relative GC-content of the plasmid constructs, fragments of *shc* were inserted into pUC18 for amplification and mutagenesis by PCR. After restriction with *Dpn*I to get rid of the parental DNA, and transformation in *E. coli* XLI Blue, plasmids were isolated and the mutations were confirmed by sequence analysis. For expression of *shc*, the mutated fragment was reintegrated into a

*Present address: Institute of Clinical Biology, Faculty of Chiba, 1-33-129 Yayoi-cho, Inage-ku, Chiba-shi, Chiba, 263-8522, Japan. Fax: +81-(0)43-290-2898.
E-mail address: christine.fuell@web.de (C. Füll).

Abbreviations: SHC, squalene–hopene cyclase

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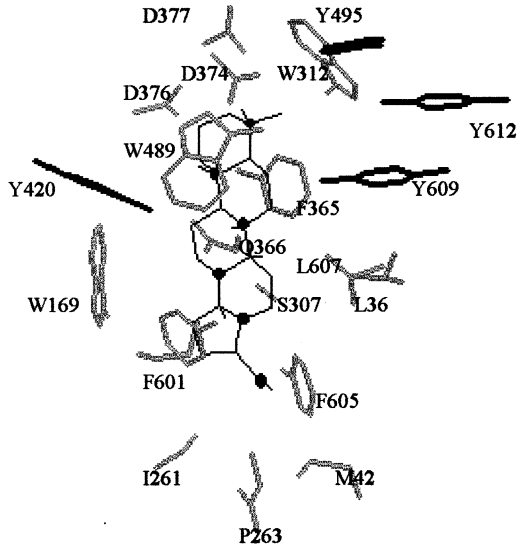

Fig. 1. Residues lining the catalytic cavity of SHC in a distance of 6 Å from the modelled hop-22(29)-ene molecule. Intermediate carbocations (C4, C10, C8, C13, C17 and C22; starting from top) in hopene skeleton are indicated as black dots. Y420, Y495, Y609 and Y612 are drawn in black.

deleted *shc* in the expression vector pKK223-3. The organism *shc* was expressed in was *Escherichia coli* JM83.

2.2. Enzyme preparation

Bacteria were grown overnight in 5 ml Lennox L Broth with 100 μg ampicillin ml^{-1} at 37°C and transferred to 800 ml of the same medium. Expression of the enzyme was induced at OD_{578} 0.5 by the addition of 80 mg isopropylthiogalactoside. Bacteria were harvested after 6 h at OD_{578} 1.6–1.7, which corresponded to 0.65 mg protein ml^{-1}, by centrifugation at 8000 rpm for 10 min, washed with saline and disrupted by osmotic shock and sonification. The enzyme was solubilized from the membrane fraction by Triton X-100 as described in [16]. The enzyme preparations were enriched about 80× and not pure. Therefore, it was not possible to measure k_{cat}.

2.3. Enzyme assay and identification of new products

The assay mixture contained 800 μl of the solubilized membrane fraction corresponding to a constant amount of protein, 100 μl Na-citrate buffer, pH 4.9, and 100 μl solubilized squalene as described in [16]. The assays were carried out at 50°C for 20 min or 16 h for mutants with a low reaction velocity. The reaction was stopped by extraction with 3 ml hexane/propan-2-ol (3:2 by vol.). Triton X-100 was adsorbed on a silica column (10×1 cm). Substrate and products were eluated with dichloromethane and evaporated to dryness. For mass spectral analysis (model Shimadzu GC 17A, MS QP 5000) the substances were dissolved in 20 μl hexane/propan-2-ol (3:2 by vol.) and severed using the following program: 3 min at 90°C, 25°C min^{-1} to 200°C, 4°C min^{-1} to 300°C, 10 min at 300°C. The compounds were identified by their fragmentation pattern according to [4,9,17,18] and the papers cited there.

3. Results and discussion

Mutants were constructed which substituted tyrosine residues at positions 420, 495, 609 and 612 as listed in Table 1. The initial velocity as well as the product pattern of these mutants were measured at 50°C and is shown in Table 1.

In a preceding paper [6] we demonstrated that SHC mutant Y609F displayed a nearly wild-type initial rate and an altered product pattern with hopene as main product, a low percentage of α-polypodatetraene and significant amounts of tetracyclic compounds. It was therefore of interest to introduce even smaller residues at this position. Especially, we wanted to compare the meaning of the aromatic ring and the polarity of the hydroxyl group. To produce an ingenious succession, we took the three non-aromatic amino acids serine, cysteine and leucine, which have some similarity in structure but reduced polarity in the series as mentioned above. The three mutants Y609C, Y609L and Y609S form γ-polypodatetraene as main product (Fig. 2). These mutants and L607K [10] represent a class of mutants with this bicyclic compound as main product. By comparison of the product patterns of the above mentioned mutant cyclases, SHC Y609L formed, in addition to the main product γ-polypodatetraene, the highest

Table 1
Initial velocity and product pattern of wild-type and mutant SHCs

	Initial velocity	Products							
		2	3	4	5	6	7	8	9
wt	100	*	1.0	*	1.3	0.7	0.8	1.0	95.2
Y420A	90.2	10.3	–	–	–	–	–	–	89.7
Y420C	<0.1	–	–	–	–	–	–	–	100
Y420F	90.2	10.3	–	–	–	–	–	–	89.7
Y420G	0.1	15.9	–	–	–	–	–	–	84.1
Y420K	0.6	–	–	–	–	–	–	–	100
Y420R	0.2	–	–	–	–	–	–	–	100
Y420S	0.2	–	–	26.1	–	–	–	–	73.9
Y495L	54.6	2.5	–	–	–	1.9	–	4.1	93.7
Y609A	0.1	–	–	–	–	–	–	–	100
Y609C	5.2	–	–	72.3	–	–	–	–	27.7
Y609F	100	4	13	–	14	2	2	14	50
Y609K	<0.1	–	–	–	–	–	–	–	100
Y609L	3.8	–	6.5	42.9	8.5	1.6	1.2	10.3	30.2
Y609R	<0.1	–	–	–	–	–	–	–	100
Y609S	7.6	–	0.8	70.1	4	–	–	3.6	21.6
Y612L	41.3	–	1.8	4.2	–	–	–	4.9	89.1

The numbers in the heading refer to the sequence of appearance in the GC-spectra: 2: α-polypodatetraene; 3: dammara-13(17),24-diene; 4: γ-polypodatetraene; 5: 17-isodammara-12,24-diene; 6: eupha-7,24-diene; 7: dammara-20(21),24-diene; 8: 17-isodammara-20(21),24-diene; 9: hopene. The structure of the compounds is given in Fig. 2. The initial velocity as a unit of measurement of relative activity was directly calculated from the areas of substrate and product peaks in the GC-spectra. Mutants Y420A and Y609F have previously been published [6,8]. Every assay was repeated at least three times and the mean value is given. The side products are calculated as a percentage of all products formed. *Traces of these compounds have been identified using GC/MS; wt stands for the wild-type enzyme.

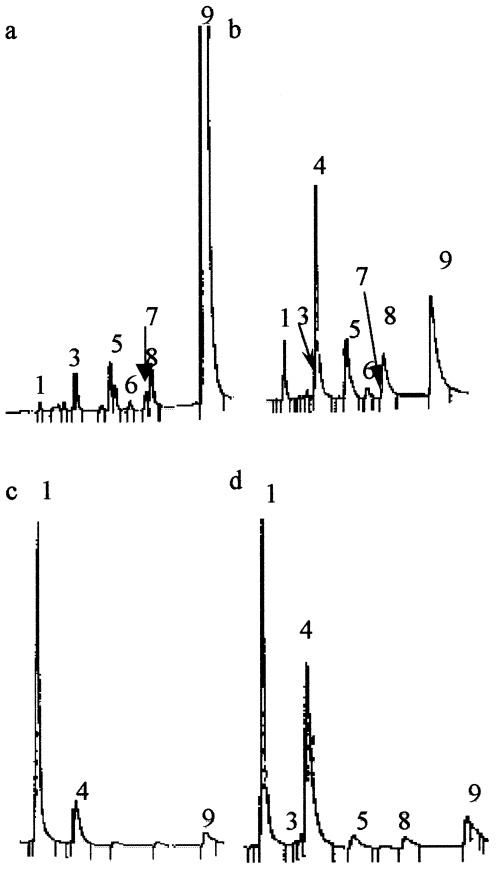

Fig. 2. GC-spectra of wild-type SHC (a/ 20 min) and mutants Y609L (b/ 16 h), Y609C (c/ 20 min) and Y609S (d/ 20 min). The times in the parentheses stand for the time of incubation. 1: squalene; 3: dammara-13(17),24-diene; 4: γ-polypodatetraene; 5: 17-isodammara-12,24-diene; 6: eupha-7,24-diene; 7: dammara-20(21),24-diene; 8: 17-isodammara-20(21),24-diene; 9: hopene.

proportion of hopene and a higher proportion of additional products, suggesting that it displays the most unspecific catalysis. Y609L seems most familiar to Y609F in this series of mutants. A possible explanation for this behavior is the additional space in the cavity combined with the complete loss of polarity of this amino acid residue. The resulting space directs the formation of two six-membered rings. The rest of the squalene chain seems misfolded and therefore becomes not cyclized. While leucine has no polarity to stabilize a carbocation, serine and cysteine are able to do so, and therefore, these mutants have a higher product specificity. In the case of leucine, other amino acid residues as F365 or Q366 are predicted to support the formation of γ-polypodatetraene by stabilizing C8.

This stabilizing effect should be important for the formation of the main product γ-polypodatetraene, because it is the thermodynamically favored product (Zajcev product). Compared to the kinetically favored product α-polypodatetraene (Hofmann or Markovnikov product), the polypodatrienyl cation needs to be stabilized when the molecule is transformed into the energetically favored γ-polypodatetraene, because the reaction velocity is lower (Fig. 3).

Interesting was the fact that the mutation Y420S had similar effects compared with Y609S on the product pattern. Residues Y420 and Y609 point from opposite sites into the cavity, and therefore, both residues may have a stabilizing effect on the C8 cation, resulting in mutants with similar properties. Y420S produced 26% γ-polypodatetraene and the reaction was slow. Mutant Y420C was almost inactive, producing solely trace amounts of hopene. As with Y609S and Y609C, the difference in the reaction velocity may be attributable to the different properties of oxygen and sulfur.

Another mutant with a very low reaction velocity was Y420G. This mutant SHC produced 16% α-polypodatetraene, and therefore confirmed the interpretation that the production

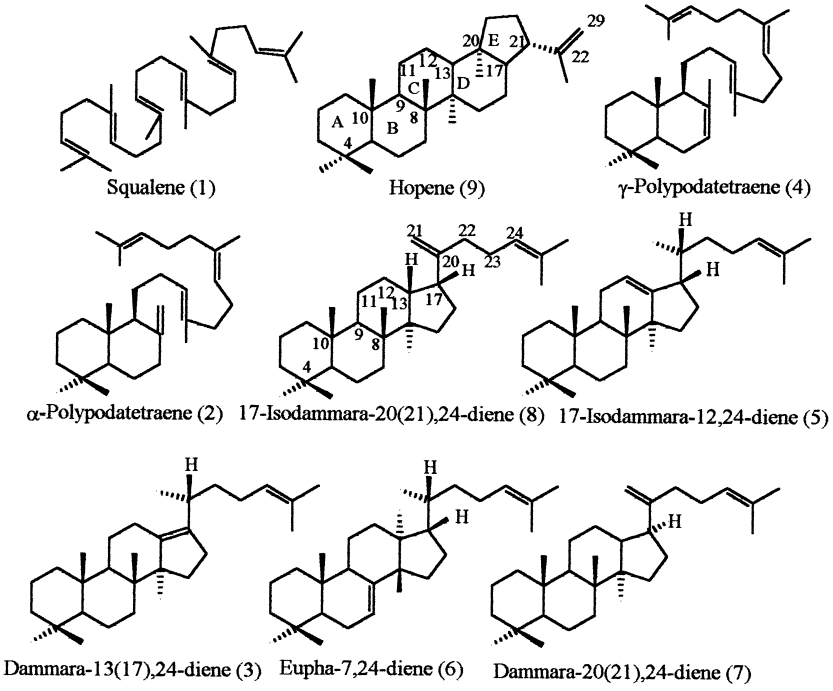

Fig. 3. Substrate and products of wild-type and mutant SHC. Numbers in the parentheses refer to their sequence in the GC-spectra (Fig. 2) and correspond to the numbers in Table 1.

of polypodatetraenes, especially α-polypodatetraene, is primarily a matter of misfolding as a consequence of additional space in the reaction cavity. Furthermore, glycine has no opportunity to stabilize a carbocation, and this is why the kinetically favored product is formed. The polypodatrienyl cation is far away from any group, that may stabilize the C8 cation. It is not surprising that the reaction rate decreased badly, because glycine is known to disrupt structural motifs, so the SHC may be destabilized by inserting this amino acid in the 'upper' part of the cavity.

Insertion of a basic residue such as lysine or arginine at the positions 420 and 609 was expected to result in the formation of a monocyclic product, but this was not observed. The mutants Y420K, Y420R, Y609K and Y609R had very low turnover rates, and the only product which could be detected was hopene. Possibly, these residues reached too far into the cavity and squalene contacted only ocassionally the protonation site [5]. It is also very possible, as we found out in a simulation with the computer model (Swiss Prot), that the basic residue built a salt bridge with the protonation side and blocked the reaction this way.

Interesting is the comparison of the published mutant Y609F [6] and mutant Y420F. Both tyrosines are located in the wall of the catalytic cavity in a mirror-image position, thereby, pointing to ring B of hopene [2,3]. This is why one expects similar effects by mutational exchange of these residues. The initial velocity of both mutants is about the same. Mutant Y420F produced a low amount of α-polypodatetraene besides hopene as main product. Mutant Y609F on the other hand showed as main effect the production of tetracyclic compounds in significant amounts (Table 1) and just a small rate of α-polypodatetraene. It seems that in Y420F, a weak stabilization for the carbocation at C8 is introduced, whereas for the rest of the carbocations, a normal stabilization remains. In contrast, the mutant Y609F shows a weaker stabilization for two carbocations (C8 and C13). The production of all side-products follows the rule of Markovnikov and results in the kinetically favored ones.

The properties of mutants Y609A, C, L, S show clearly that the residue at position 609 has to be larger than alanine. In all cases, the initial velocity is low. It is enhanced to wild-type velocity in Y609F, but at low specificity. Taken together, we were able to show that the position 609 is a key position in the catalytic cavity concerning both the reaction velocity as well as the product pattern. In contrast, the position 420 leaves more space for variation of amino acid residue.

The other two tyrosine residues that were mutated in this study function in the initiation of the reaction, as previously shown [6] and predicted in [3]. The mutations Y495L and Y612L showed a moderate activity and had a wild-type product pattern. The initial velocity of both mutants was higher than that of their corresponding phenylalanine mutants [6], which means that leucine leaves more space in this area to allow another amino acid to move into the right position to compensate the effect of the missing hydroxyl group. With these findings we were able to confirm some of the hypotheses of previous mechanistic [4] and structural [3] studies.

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