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Point mutation of (+)-germacrene A synthase from *Ixeris dentata*

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Abstract

Sesquiterpene cyclases catalyze the conversion of common precursor, farnesyl pyrophosphate, into various terpene backbones. X-ray crystallography of tobacco *epi*-aristolochene synthase has previously proposed a cyclization mechanism wherein the allylic carbocation intermediate is stabilized by the main chain carbonyl oxygens of three consecutive threonine residues. Alignment of amino acid sequences of plant terpene cyclases shows that the first position of the triad is almost invariably threonine or serine. To probe the carbocation-stabilizing role, the amino acid residues of the $_{433}$TSA$_{435}$ triad in (+)-germacrene A synthase from *Ixeris dentata* were altered by site-directed mutagenesis. Enzyme kinetic measurements of the mutants and GC/MS analysis of the enzyme reaction products indicate that mutations of the triad decreased enzyme catalysis rather than substrate binding but did not affect its structural rearrangement in the catalytic mechanism. This is the first report that the hydroxyl group of threonine at the first position of the triad is required for the cyclase activity.

Introduction

Sesquiterpene cyclases or synthases convert farnesyl pyrophosphate (FPP) into various terpene backbones (Sacchettini & Poulter 1997). Nevertheless, they all share common structural motif facilitating departure of the pyrophosphate group from FPP and stabilizing the carbocation intermediate (Lesburg *et al.* 1998) (Figure 1). One of the well-characterized sesquiterpene synthases of plant origin is *epi*-aristolochene synthase (TEAS) from tobacco, its structure being solved by X-ray crystallography at 2.2 Å resolution (Starks *et al.* 1997). In the proposed mechanism, the main chain carbonyl oxygens of residues T_{401} and T_{402} and the hydroxyl group of T_{403} are suggested to stabilize the allylic carbocation intermediate. These dipoles direct the cationic end of the farnesyl chain into the hydrophobic active site.

In this context, the amino acid sequences of various plant terpene cyclases were aligned at 401–403 triad positions of TEAS (Table 1). The position corresponding to 401 of TEAS is almost invariably threonine or serine, an amino acid with 3-hydroxyl group. On the other hand, the second and the third positions are varied with amino acids of neutral or hydrophilic side chain. The sequence alignment suggests that the nature of amino acid residue at the first position should be important for the cyclase activity.

To probe their carbocation-stabilizing role and support the creation of novel terpene cyclase, the amino acid residues at corresponding positions 433–435 on (+)-germacrene A synthase from *Ixeris dentata* (IdGAS, Genbank AY082672) (Kim *et al.* 2005) were altered by site-directed mutagenesis. The probable role of the positions was suggested through enzyme kinetic measurements of the mutants and GC/MS analysis of the enzyme reaction products.

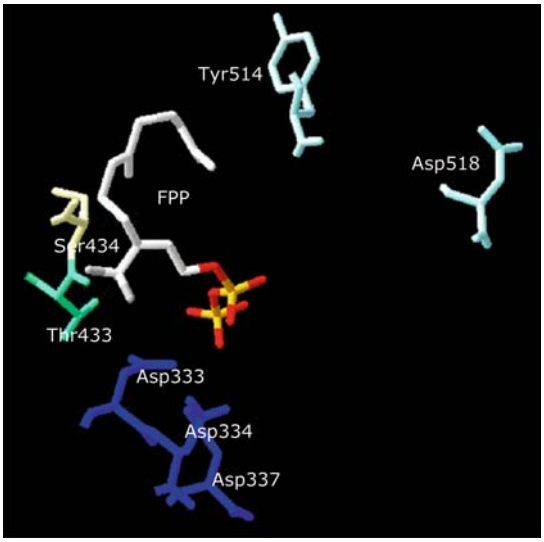

Fig. 1. Schematic drawing of IdGAS active site based on X-ray crystallography of TEAS. Magnesium ions (not shown) bound to Asp_{333} and Asp_{337}, a part of DDXXD motif for terpene cyclase, were proposed to facilitate the departure of pyrophosphate group (orange & red) from FPP substrate. The catalytic residues, Tyr_{514} and Asp_{518}, were positioned on the interior surface of the active site. In this work, it is proposed that the hydroxyl group of Thr_{433} with a little help of Ser_{434} stabilizes and directs the cationic end of the farnesyl chain into the hydrophobic active site.

Materials and methods

Site-directed mutagenesis

PCR amplification for site-directed mutagenesis (Sambrook & Russell 2001) was done with 500 pg *IdGAS* plasmid (Kim *et al.* 2005), 0.5 mM each primer, and 1.25 units *Pfu* DNA polymerase (MBI Fermentas) in 50 μl reaction buffer composed of 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1 mM each dNTP, 0.1 mg ml^{-1} BSA, and 0.1% Triton X-100. Oligonucleotide 34-mer primers containing the desired mutation (Table 2) were synthesized by Bioneer (Daejon, Korea). Sequences of the other PCR primer were inverse complementary to the primer sequence listed in Table 2. For double mutations, the single mutated plasmid (M1 or M3) was used for PCR template. PCR was cycled 12 times as follows: 1 min (3 min at the first step) denaturation at 94 °C, 1 min annealing at 50 °C, and 9 min (10 min at the final step) extension at 72 °C. Endonuclease *Dpn* I-treated PCR product was used for transformation of bacterial cells. For the confirmation of mutagenesis, the mutated plasmids were sequenced in both directions.

Expression and enzyme purification

Wild type and mutant cells were cultured in 1 l LB medium supplemented with ampicillin (50 μg ml^{-1}) and induced with 0.6 mM IPTG. The cell pellet in 5 ml lysis buffer composed of 100 mM Tris/HCl (pH 8), 1 mM PMSF, 10% (v/v) glycerol, and 5 mM $MgCl_2$ was disrupted by sonication (Sonic & Materials VC-600 microprobe) at maximum power for 30 min with 20 s burst followed by 20 s chilling on the ice. The soluble protein was partially purified with ProBand resin (Invitrogen) and further purified with FPLC (Pharmacia) employing Mono-Q column with gradient elution from 0.15 to 0.3 M NaCl in 50 mM sodium phosphate

Table 1. List of the corresponding triad site in sesquiterpene cyclases.

Triad	Sesquiterpene Cyclase	Source	GenBank no.
TTT	5-*epi*-Aristolochene synthase	*Nicotiana tabacum*	L04680
TSA	Germacrene A synthase	*Ixeris dentata*	This work
TST	Vetispiradiene synthase	*Lycopersicon esculentum*	AF171216
TGG	Amorphadiene synthase	*Artemisia annua*	AJ251751
TCG	δ-Cadinene synthase	*Gossypium arboreum*	AF174294
TSA	Germacrene A synthase	*Lactuca sativa*	AF489964
TGG	Germacrene D synthase	*Solidago canadensis*	AY397645
SAG	Germacrene C synthase	*Lycopersicon esculentum*	AF035631
SSG	β-Caryophylene synthase	*Artemisia annua*	AF472361
SSG	δ-Selinene synthase	*Abies grandis*	AF326513
SIG	8-Epicedrol synthase	*Artemisia annua*	AF157059
SIG	α-Bisabolene synthase	*Abies grandis*	AF006195

Table 2. List of PCR templates and primer sequences for site-directed mutagenesis.

IdGAS	Mutation[a]	Template	Primer (34-mer)[a]
WT	TSA	–	GAAGAACGGGTTGATTACTTCGGCTTATAATGTC
M1	ASA	WT	GAAGAACGGGTTGATTGCTTCGGCTTATAATGTC
M2	AST	M1	GAAGAACGGGTTGATTGCTTCGACTTATAATGTC
M3	TST	WT	GAAGAACGGGTTGATTACTTCGACTTATAATGTC
M4	TAA	WT	GAAGAACGGGTTGATTACTGCGGCTTATAATGTC
M5	ATA	M1	GAAGAACGGGTTGATTGCTACTGCTTATAATGTC
M6	AAT	M1	GAAGAACGGGTTGATTGCTGCGACTTATAATGTC
M7	AAA	M1	GAAGAACGGGTTGATTGCTGCGGCTTATAATGTC
M8	TTT	M3	GAAGAACGGGTTGATTACTACTACTTATAATGTC

[a]Mutation sites are underlined. Sequences of the other PCR primer, omitted, were inverse complementary to the primer sequence listed above.

(pH 7). Protein concentrations were determined through the Bradford method using BSA as the standard. SDS-PAGE on 10% (v/v) running gel was stained with Coomassie Brilliant Blue.

Enzyme reaction

For the activity test, ProBand-purified enzyme (1.2 mg protein) was mixed with 1 ml reaction buffer containing 45 mM HEPES, 4.5 mM β-mercaptoethanol, 4 mM DTT, 5.5 mM $MgCl_2$ (pH 7) and 40 μg farnesyl pyrophosphate (FPP) (Sigma,). The enzyme reaction was overlayed with 5 ml hexane and incubated at 25 °C for 3 h. The reaction product was extracted with hexane three times. The extracts were passed through a silica gel column and concentrated under stream of N_2 prior to GC/MS analysis. For the kinetic study, FPLC-purified enzyme (0.05–1 μg protein in 50 μl) was used with a substrate of 0.15 μCi (1-^{3}H)FPP (16.1 Ci mmol^{-1}, NEN, Boston) diluted with 5–80 mM cold FPP. The reaction mixture was incubated at 25 °C for 30 min and added with 710 μl 0.5 M EDTA to stop the reaction. Reaction mixture was extracted with 1 ml hexane and treated sequentially with silica gel and anhydrous $MgSO_4$. Hexane phase (500 μl) was mixed with 3 ml LSC cocktail (ScintiVerse I, Fisher Scientific) and measured by liquid scintilation counter (Wallac 1409).

GC/MS analysis

HP5890 series II system (Germany) equipped with an AX505WA mass spectrometer (JEOL, Japan) operating in an EI (electron impact) mode was used under the condition as follows: injector temperature, 250 °C; flow rate, 2 ml min^{-1}; N_2 carrier gas; split ratio, 5:1; DB-5 column, 30 m × 0.25 μm × 0.25 mm; oven temperature, held at 100 °C for 2 min, 5 °C min^{-1} increased to 150 °C, 10 °C min^{-1} increased to 250 °C, and held at 250 °C for 2 min (Chang *et al.* 2000).

Results and discussion

The main chain carbonyl oxygen at three consecutive threonine residues plays a role in stabilizing the carbocation intermediate in TEAS (Starks *et al.* 1997). As the backbone carbonyl group is common to any amino acid residue, any amino acid could replace threonine residue from the first position for the sesquiterpene cyclase activity. Therefore, triad of the wild-type IdGAS, $_{433}$TSA$_{435}$ corresponding to $_{401}$TTT$_{403}$ of TEAS was altered by site-directed mutagenesis to find out the role of the positions.

Enzyme kinetics of IdGAS mutant M1 (ASA) and M2 (AST) showed the decrease of k_{cat} about by two orders. However, M3 (TST) recovered full activity comparable to the wild type (Table 3). For a throughput screening of threonine in these three positions, complete combination of sequences with two alanines and one threonine generated three mutants M4, 5 and 6. Only mutant M4 (TAA) had the same activity as the wild type, while mutant M5 (ATA) and M6 (AAT) had no activity at all. Mutant 7 (AAA), absence of threonine in any position, had no activity as well. These data indicate that the sesquiterpene cyclase activity is dependent on threonine at the first position of the triad. The hydroxyl-group side chain of threonine

Table 3. Enzyme kinetics of IdGAS mutants.

IdGAS	Mutation	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
WT	TSA	11	1.6×10^{-2}	1.5×10^{-3}
M1	ASA	4.7	3.5×10^{-4}	7.5×10^{-5}
M2	AST	7.6	3.7×10^{-4}	4.9×10^{-5}
M3	TST	25.1	4.4×10^{-2}	1.7×10^{-3}
M4	TAA	10.6	1.7×10^{-2}	1.6×10^{-3}
M5	ATA	–	No activity	–
M6	AAT	–	No activity	–
M7	AAA	–	No activity	–
M8	TTT	–	No activity	–

residue should play a role in carbocation stabilization, which contradicts the role of main chain carbonyl oxygen (Starks *et al.* 1997).

However, it was unexpected that Mutant 8 (TTT) had no activity. It is not evident yet why M8 was inactive while triad TTT in native TEAS confers the cyclase activity. Because M3 (TST) had full activity comparable to the wild type (TSA), it is possible that three threonine residues in M8 would introduce a steric hindrance near the active site of IdGAS. This relationship was again found between M5 (ATA) and M1 (ASA). Mutant M1, absence of threonine at the first position, still retain marginal activity provided the second position was sterically less demanding serine compared with threonine. Therefore, it is possible that the hydroxyl group on S_{434} of M1 manages to stabilize the carbocation while the bulkier hydroxyl group of threonine at the same position in M5 cause the steric hindrance and no detectable activity.

Enzyme kinetics of IdGAS mutants, on the other hand, are in agreement with the proposed mechanism (Starks *et al.* 1997) that the stabilization of the carbocation intermediate might be related with enzyme catalysis rather than substrate binding. The K_m ratios between the mutant and the wild type varied up to three-fold more or less, while k_{cat} ratios were about two orders. In overall, these point-mutations were effective more on enzyme catalysis (k_{cat}) and efficiency (k_{cat}/K_m) than on substrate binding (K_m).

When the enzyme products of hydrocarbons were assayed by GC/MS, no hydrocarbon other than germacrene A was detected in any IdGAS mutants. Therefore, mutation of the hypothetical carbocation-stabilizing triad did not affect the course of structural rearrangement of carbocation on the enzyme hydrophobic groove. This is the first report emphasizing the importance of hydroxyl group of threonine at the first position for the cyclase activity.

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