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Domain–Domain Interactions in the Iterative Type I Polyketide Synthase ATX from *Aspergillus terreus*

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Fungal aromatic polyketides are biosynthesized by iterative type I polyketide synthases (iPKSs), which consist of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains as a minimum, with additional domains such as ketoreductase (KR), methyltransferase (MeT), and Claisen cyclase (CYC), all on single polypeptides. These catalytic domains are involved in repetitive Claisen condensations to form β-polyketomethylene intermediates, which are then cyclized and aromatized by aldol and/or Claisen cyclization.[1] Although no three-dimensional data have been reported on iterative type I PKSs so far, recent X-ray crystallographic studies unveiled the structures of modular type I polyketide synthase (PKS) fragment, the KS-AT didomain from DEBS module 5,[2] type II actinorhodin KS-chain length factor (CLF) heterodimer,[3] type III PKSs,[4] and mammalian and fungal type I fatty acid synthases (FASs).[5,6] All these PKSs are found as "head-to-head and tail-to-tail" dimers except fungal FAS that form $\alpha_6\beta_6$ heterododecamers.[6] From these facts, the "head-to-head and tail-to-tail" dimer is considered to be the basic architecture common in PKS and FAS proteins.

Biosynthesis of fungal tetraketide aromatic compounds has long been studied intensively. 6-Methylsalicylic acid (6-MSA) is the landmark compound used to establish the polyketide pathway, and its synthase (MSAS) from *Penicillium patulum* was the first PKS to be purified.[7–11] Recent molecular genetic studies revealed genes for MSAS from a number of fungal sources, and heterologous expression systems of these genes have been established.[12–14] As an iPKS, MSAS is the smallest in size, approximately 190 kDa, consisting of KS, AT, dehydratase (DH), KR, and ACP domains. Its domain organization is quite similar to that of mammalian FAS, although MSAS lacks enoyl reductase (ER) and thioesterase (TE) domains. It has been assumed that KS, AT, and ACP domains are for starter loading and following condensations, and KR and DH domains are for introduction of double bonds after the second condensation. Although no mutation studies on MSAS catalytic domains, except KR, have been carried out, our previous deletion experiments on ATX, a MSAS from *Aspergillus terreus*, indicated that the conserved KS domain and helical structures at both N and C termini could be essential for its activity. By further deletion analysis, we identified the ID region, which is the minimum region between the DH domain and KR domain required for subunit–subunit interactions in ATX (Figure 1).[15] As MSAS is known to be a homotetramer,[10,11,15] we proposed a model for ATX higher-level structure, considering that the "head-to-head

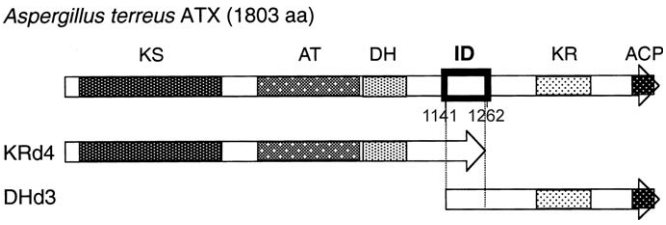

Figure 1. ATX deletion mutants with the minimum length needed to reconstitute the catalytic reaction center by coexpression. The ID region is required for ATX subunit–subunit interaction to form dimeric or tetrameric active catalytic centers.[15] KRd4 is the minimal mutant with C-terminal deletion and DHd3 is the minimal mutant with N-terminal deletion for reconstitution of the reaction center by coexpression in yeast.

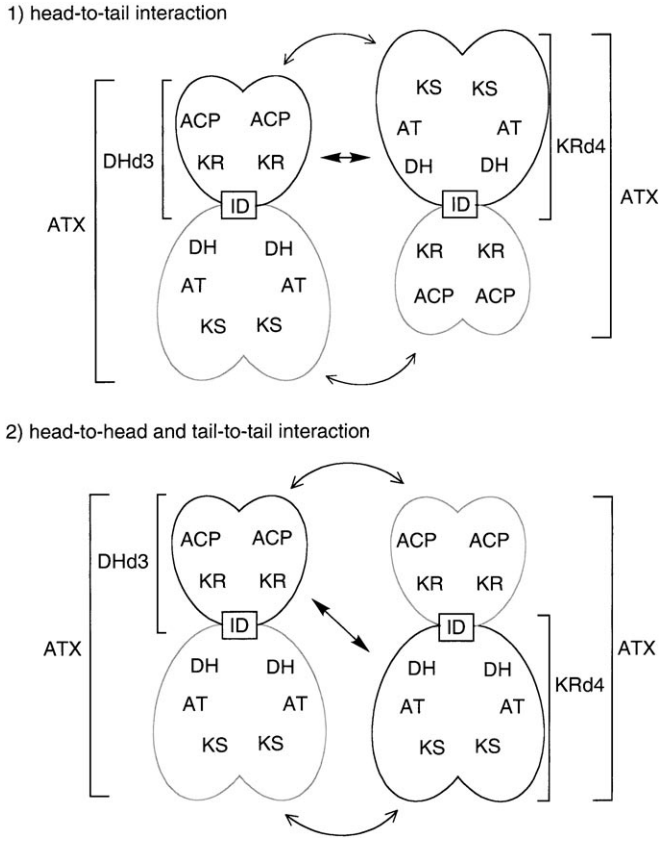

Figure 2. Proposed ATX dimer–dimer interaction models from deletion mutant analyses. "Head-to-head and tail-to-tail" dimers may be formed by interaction of ID regions and the two dimers may interact either in a "head-to-tail" or "head-to-head and tail-to-tail" manner.

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and tail-to-tail" dimer is the basic architecture in PKSs. As shown in Figure 2, the "head-to-head and tail-to-tail" dimer of the ATX subunit may interact either in a "head-to-tail" manner or "head-to-head and tail-to-tail" manner to form a catalytically active reaction chamber.[15]

For further analysis of domain functions and their interactions in the ATX reaction center, we first carried out expression of ATX catalytic domain mutants in yeast. Conserved domain searches easily indicated the presence of KS, AT, KR, and ACP domains but failed to show a DH domain in MSAS. The DH domain region of ATX was assigned by comparison of secondary structural elements predicted by PredictProtein http://www.predictprotein.org/ with the discrete DHs of type II FAS from *E. coli* that have three helices and seven sheets in a "hotdog" fold.[16] The consensus sequence of the DH domain, HxxxGxxxxP, has been identified in FAS, of which the histidine residue is the active center for dehydration,[17] and the corresponding sequence ^{972}HxxxGxxxxP^{981} was found in ATX. The discrete DHs, such as type II FAS from *E. coli*[16] and *Pseudomonas aeruginosa*[18] have been studied and their higher structures were solved by X-ray crystallographic analysis. However, only mutation analysis was reported on the DH domain of modular PKS, that is, the DH domain of the picromycin/methymycin PKS (PICS) module 2. PICS module 2(DH^0) + TE, with an inactivated DH domain, produced exclusively undehydrated hydroxyl product.[19] On iPKSs, no functional analyses of their DH domains have been carried out including MSAS. It is known that bacterial discrete DHs catalyze the introduction of *trans* double bonds, but the actual function of MSAS DH domain is unknown whether DH actually catalyzes dehydration of the β-hydroxyl triketide intermediate after KR reduction to directly form the *cis* double bond which is required for cyclization and aromatization in MSAS reaction.

To express ATX catalytic domain mutants, point mutations were introduced into the wild-type *atX* cDNA by overlap PCR with specific primers and the mutated *atX* fragments were inserted downstream of the GAL10 promoter of pESC yeast expression vectors as previously reported for ATX deletion mutant expression.[15] Then, a transformant of each mutant was cultured in induction medium with galactose. ATX KS domain mutant C216A (KSm) could not produce 6-MSA. Similarly, AT domain mutant S667A (ATm) and ACP domain mutant S1761A (ACPm) lost 6-MSA production ability, as expected. Thus, the crucial roles of these catalytic domain residues were confirmed for ATX 6-MSA synthesis.

In the KR domain, a consensus sequence GxGxxG was mutated to AxPxxA according to the mutational analysis on the KR domain of *P. patulum* MSAS.[20] The ATX KR domain mutant ^{1441}AxPxxA^{1446} (KRm) produced triacetic acid lactone (TAL) as reported previously.

To analyze the DH domain function, ATX H972A mutant (DHm) was constructed and expressed in yeast. As dehydration was believed to occur just after the reduction of the triketide intermediate in the MSAS reaction, production of triketide derivative by DHm was expected. However, HPLC analysis of DHm induction culture could not detect production of such a compound. A DH-like sequence (^{939}HxxxGxxxP^{947}) is also conserved among MSASs and the ATX H939A mutant could produce 6-MSA (data not shown). This result indicated that His^{972} is crucial for ATX reaction not just for simple dehydration. If dehydration could occur immediately after the reduction step of the triketide intermediate by KR, production of TAL would be expected with DHmKRm, that was a double mutant in both DH (H972A) and KR (^{1441}AxPxxA^{1446}) domains. However, production of TAL was not observed in DHmKRm expression culture as shown in Figure 3.

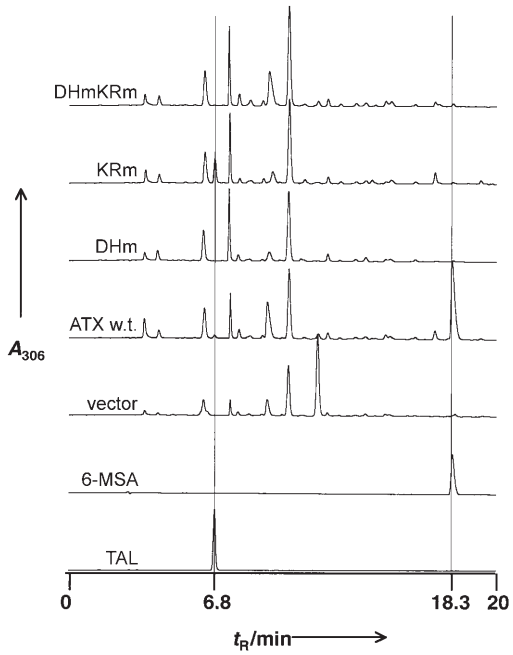


Figure 3. HPLC analysis of 6-MSA and TAL production by yeast transformants expressing ATX domain mutants. DHm, DH domain mutant; KRm, KR domain mutant; DHmKRm, DH and KR domains double mutant. HPLC was eluted with a linear gradient of H_2O/CH_3CN from 90:10 to 40:60 over 23 min monitored at 306 nm. Triacetic acid lactone (TAL) and 6-methylsalicylic acid (6-MSA) were eluted at $t_R = 6.8$ min and 18.3 min, respectively.

These results suggested that the ATX DH domain should function as more than a simple dehydratase of a β-hydroxyl triketide intermediate in 6-MSA formation. Interestingly, the DH motif HxxxGxxxxP is also found in the bacterial orsellinic acid synthases, *Streptomyces viridochromogenes* AVIM[21] and *Micromonospora echinospora* CalO5,[22] although no apparent dehydration is involved in orsellinic acid synthesis reaction. Neocarzinostatin naphthoate synthase (NNS) from *Streptomyces carzinostaticus* was proposed to catalyze the formation of 2-hydroxy-5-methyl-1-naphthoic acid by two rounds of KR-DH reactions.[23] Although NNS is a hexaketide synthase, it has a quite similar architecture to ATX with 39% amino acid sequence identity. Alignment of the DH domains of these iPKSs including *P. patulum* MSAS shows a highly conserved amino acid sequence. DH domains of modular type I PKSs are also highly homologoues. Although DH sequences of iPKSs, modular PKSs, and bacterial discrete DHs such as FabAs and FabZs, are not well conserved with respect to each other, all these enzymes have the HxxxGxxxxP motif (Figure 4).

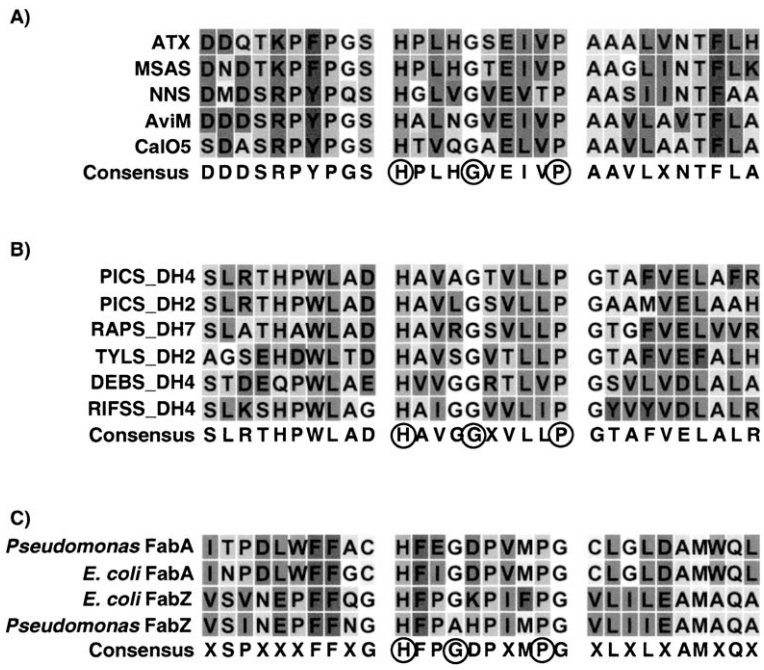
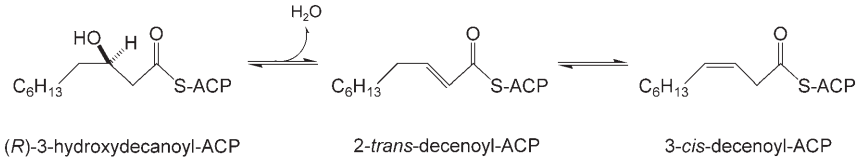


Figure 4. Amino acid sequence alignments of dehydratase domains of polyketide synthases and dehydratases of bacterial fatty acid synthases. A) ATX, aa 962-991, BAA20102; MSAS, aa 948-977, CAA39295; NNS, aa 972-1001, AAM77986; AviM, aa 930-959, AAK83194; CalO5, aa 921-950, AAM70355, B) PICS_DH4, PikAII, aa 2450-2479, Q9ZGI4_9ACTO; PICS_DH2, PikAI, aa 3601-3630, Q9ZGI5_9ACTO; RAPS_DH4, RapB, aa 4143-4172, Q54296_STRHY; TYLS_DH2, TylG, aa 3536-3565, O33954_STRFR; DEBS_DH4, EryAII, aa 2399-2428, Q5UNP5_SACER; RIFS_DH4, RifB, aa 945-974, O52545_AMYMD, C) *Pseudomonas* FabA, aa 60-89, AAC45619; *E. coli* FabA, aa 61-90, AAC74040; *E. coli* FabZ, aa 44-73, AAC73291; *Pseudomonas* FabZ, aa 39-68, AAG07033. Conserved amino acids in Hx(x)xGxxxxP region are shown as circled. Alignments were generated by using CLC Free Workbench.

It has been unknown whether the triketide intermediate tethered on ACP takes the "linear" conformation or the "curved" conformation, which is the substrate of KR in the MSAS reaction. If the linear triketide could be the direct substrate and the following dehydration by DH could give a 2-*trans*-enoyl intermediate, isomerization to the 3-*cis*-enoyl form would be necessary to allow the intermediate to fit in the catalytic reaction cavity for further condensation and aldol cyclization. It was reported that FabA carries out the isomerization of 2-*trans*- to 3-*cis*-decenoyl-ACP by allylic rearrangement (Scheme 1).[24] This suggests that DH in iPKSs, including ATX, may be involved in isomerization of a 2-*trans*-enoyl intermediate to the 3-*cis*-enoyl form. It might also be possible that the ATX DH domain catalyzes the hydrolysis of 6-MSA ACP thiolester to release the product as the "hotdog" superfamily includes hydrolase and thioesterase as its main members.[25] These possible roles of the ATX DH domain are shown in the reaction scheme (Scheme 2).



Scheme 1. Isomerization reaction of 2-*trans*- to 3-*cis*-decenoyl-ACP catalyzed by *E. coli* FabA. FabA catalyzes two reversible reactions, the dehydration of (*R*)-3-hydroxydecanoyl-ACP to 2-*trans*-decenoyl-ACP and the isomerization of 2-*trans*-decenoyl-ACP to 3-*cis*-decenoyl-ACP.

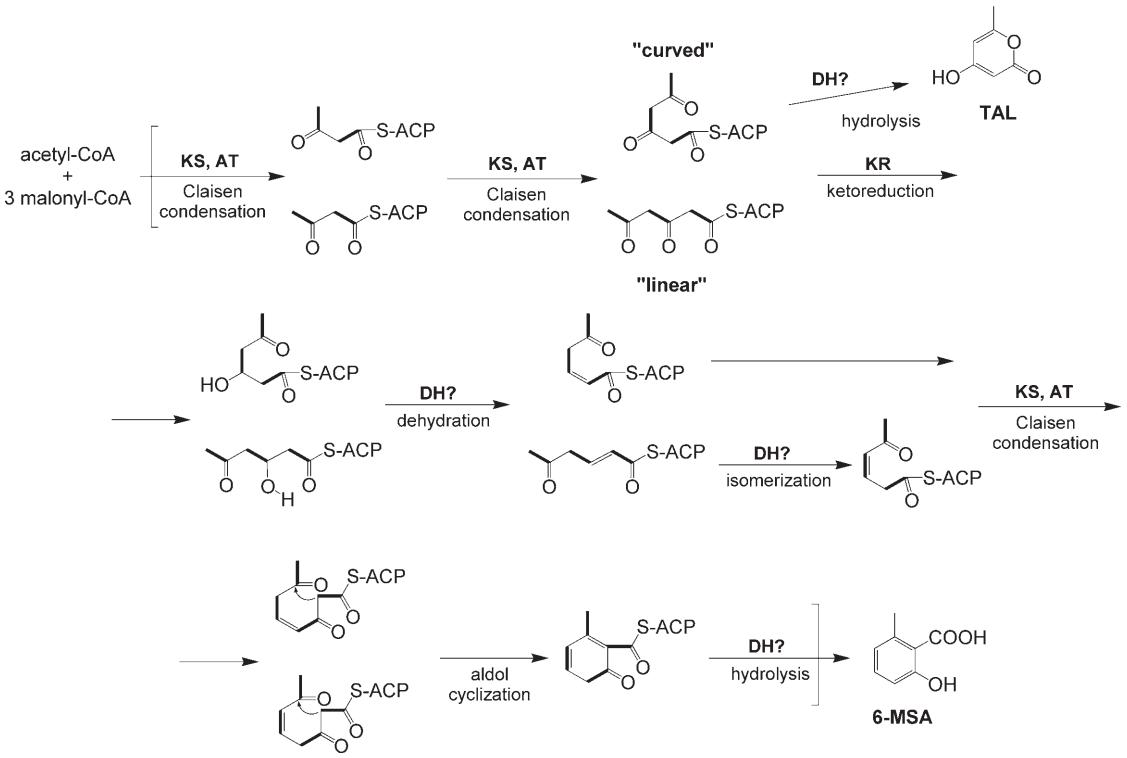
As described above, all catalytic domain mutants of ATX (KSm, ATm, ACPm, KRm, and DHm) lost 6-MSA production activity. We previously demonstrated that coexpression of inactive ATX deletion mutants with overlapping ID regions could reconstitute the catalytic reaction center for 6-MSA synthesis in yeast.[15] Using this coexpression system, these mutants were used for analysis of the catalytic domain–domain interaction. As a result, the cotransformant expressing KSm and ACPm could produce 6-MSA (Figure 5 A). Also, coexpression of DHm with either KSm or ACPm could produce 6-MSA, possibly by reconstitution of the catalytic reaction centers (Figure 5 B). This result also confirmed that DHm itself was not inactive because of secondary structure change caused by introduction of a mutation, but was active except for the DH domain. Then, coexpression of all other combinations of ATX domain mutants was analyzed and all of them could reconstitute ATX activity (Table 1).

Mammalian FAS is homodimeric, and has domain architecture similar to iPKS. In FAS mutant complementation analysis, heterodimers comprised of a subunit containing either a KS or AT domain mutant, paired with a subunit containing mutations in any one of the other five domains, DH, ER, KR, ACP, or TE, were active in fatty acid synthesis but domain mutant heterodimers in either DH, ER, KR, ACP, or TE were inactive.[26] Therefore, it had been presumed that only KSm and ATm could reconstitute the reaction center by coexpression with other domain mutants in ATX. We observed that each domain mutant could be complemented by any other kind of domain mutation to reconstitute the reaction center for the syn-

Table 1. Summary of single-domain mutant coexpression experiments.

Mutant 2: domain mutation	Mutant 1: domain mutation KS	AT	DH	KR	ACP
KS	–[b]	+[a]	+	+	+
AT		–	+	+	+
DH			–	+	+
KR				TAL	+
ACP					–

[a] +: 6-MSA was detected in the culture of the cotransformant of mutant 1 and mutant 2. [b] –: no 6-MSA was detected in the cotransformant culture. All heterologous combinations of inactivated catalytic domain mutants could reconstitute MSAS activity.



Scheme 2. Possible functions of the DH domain in the 6-MSA synthase reaction. The DH domain might catalyze not only the dehydration of the triketide intermediate but also the isomerization of an intermediate and/or the hydrolysis to release the product from ACP.

thesis of 6-MSA. In addition, when the KS domain mutant KSm was coexpressed with the four domains mutant (ATm-DHm-KRm-ACPm) the cotransformant could produce 6-MSA. This kind of complementation was observed in all other domain mutants (Figure 6).

As proposed in our previous report, the ID region could have a role in interaction of the two subunit polypeptides in a "head-to-head and tail-to-tail" manner, which further interacts in a "head-to-head and tail-to-tail" or "head-to-tail" manner. Thus, coexpression of the KS-ATm-DHm-KRm-ACPm subunit and KSm-AT-DH-KR-ACP subunit, for example, resulted in stochastic formation of active catalytic center in tetrameric ATX enzyme, that is, the N half (KS-ATm-DHm) interacts with another N half (KSm-AT-DH) and C half (KR-ACP). In the ATX catalytic center, the five active domains, KS, AT, DH, KR, and ACP, even when each was on either one of four subunits, could interact with each other with substantial flexibility and carry out 6-MSA synthesis (Figure 7).

Experimental Section

Materials: The *atX* cDNA, ATX yeast expression plasmid pESC-ATX, and SFP expression plasmid p424-SFP were constructed previously.[15] Plasmids p424 GPD was obtained from American Type Culture Collection. pESC-URA and pESC-HIS were from Stratagene. *Saccharomyces cerevisiae* INVSc1 (*MATa his3D1 leu2 trp1–289 ura3–52*) was from Invitrogen.

Construction of ATX domain mutants: The N-terminal fragment upstream of the mutation and the C-terminal fragment downstream of the mutation, or vice versa, were amplified using the intact *atX* cDNA as a template by Phusion high fidelity DNA polymerase (Finnzymes). Then, overlap PCR of these N-terminal and C-terminal fragments amplified by Phusion high fidelity with specific 5′- and 3′-terminal primers gave the full-length *atX* cDNA with mutation, which was introduced into a pESC-URA or pESC-HIS vector. Active site amino acids of all catalytic domains were mutated to alanine except for the KR domain. In the case of the KR active site, GxGxxG was mutated to AxPxxA as previously reported.[20] Additional mutations were introduced by the same overlap PCR method using mutated *atX* cDNAs as PCR templates.

Coexpression of ATX domain mutants: Transformation of *S. cerevisiae* INVSc1 was carried out with the frozen-EZ yeast transformation II™ kit (ZYMORESEARCH). Cotransformants with two different ATX mutant plasmids (pESC-URA-ATX and pESC-HIS-ATX) and SFP expression plasmid p424-SFP were selected on SC minimal plates lacking uracil, histidine, and tryptophan. After preculture in SC medium containing 2% glucose for two days at 30 °C, 220 rpm, the cotransformant was transferred into SC medium containing 2% galactose and 1% raffinose to induce *atX* expression and cultured at 30 °C, 220 rpm overnight.

HPLC analysis of the ATX product: Induction culture was extracted with an equal volume of ethyl acetate twice under acidic condition. To detect 6-MSA, evaporated residue was dissolved in 200 μL of CH_3CN and 10 μL was injected and analyzed by HPLC. HPLC analysis was performed on a Tosoh DP 8020 pump, PD 8020 photodiode array detector, and an Inertsil® ODS-3 column (4.6× 150 mm; GL Sciences, Tokyo, Japan) with a solvent system of CH_3CN containing CH_3COOH (1%, solvent B) and H_2O containing CH_3COOH (1%, solvent A) at a flow rate of 0.8 mL min^{-1}. Elution was performed with a linear gradient of solvent A/solvent B from 80:20 to 20:80 over 30 min for the detection of only 6-MSA. Under

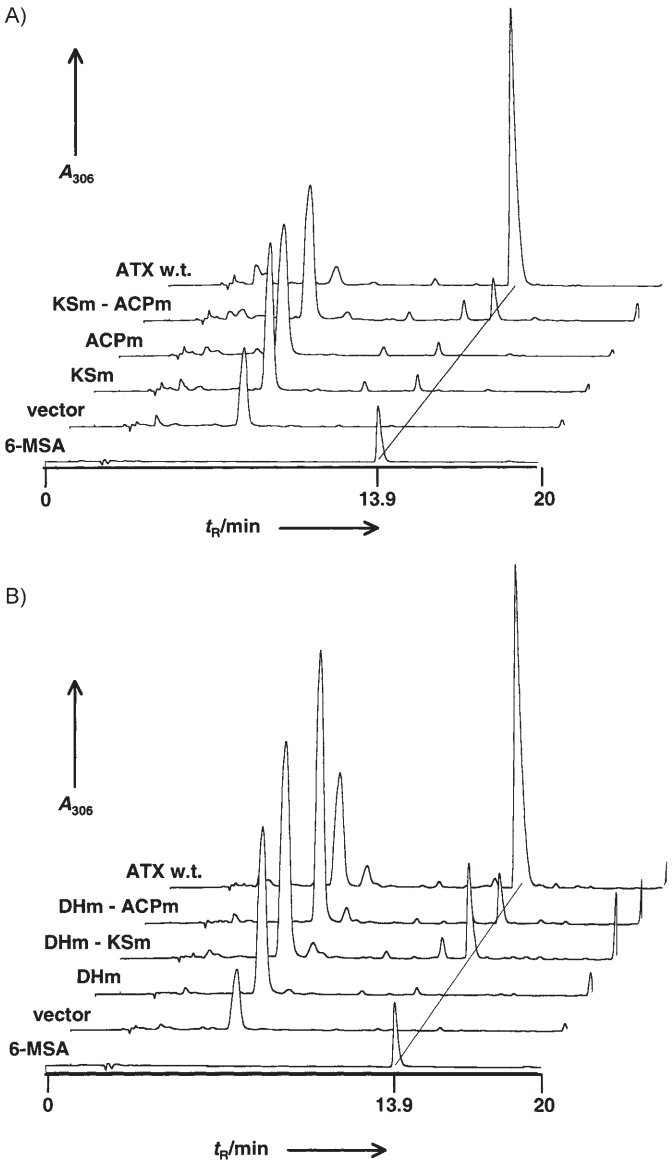


Figure 5. Coexpression of domain mutants in yeast. KSm, KS domain mutant; DHm, DH domain mutant; ACPm, ACP domain mutant; KSm-ACPm, KSm and ACPm cotransformant; DHm-KSm, DHm and KSm cotransformant; DHm-ACPm, DHm and ACPm cotransformant. A) HPLC analysis of KSm-ACPm production. B) HPLC analysis of DHm-KSm and DHm-ACPm production. HPLC was eluted with a linear gradient of H_2O/CH_3CN from 80:20 to 20:80 over 30 min monitored at 306 nm. 6-Methylsalicylic acid (6-MSA) was eluted at $t_R = 13.9$ min.

these conditions, 6-MSA was identified by comparison with an authentic sample at a retention time of 13.9 min. To observe both TAL and 6-MSA at the same time, evaporated residue was dissolved in 200 μL of 10% CH_3CN aq. and 10 μL was injected, elution was performed with a linear gradient of solvent A/solvent B from 90:10 to 40:60 over 23 min, and TAL and 6-MSA was identified by comparison with an authentic sample at a retention time of 6.7 min and 18.3 min respectively. *S. cerevisiae* INVSc1 transformant with pESC-ATX produced 6-MSA in approximately 75 $\mu g\,mL^{-1}$ culture medium, which was used as a positive control of 6-MSA production in HPLC analyses.

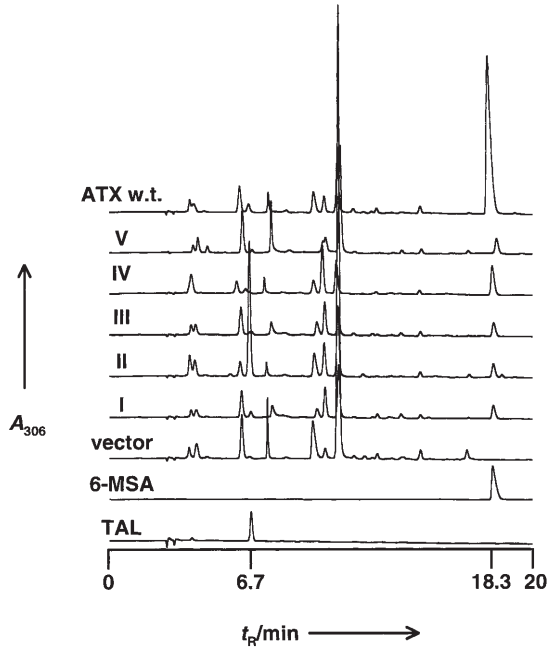


Figure 6. Coexpression of a single domain mutant and quadruple domain mutant. Every single domain mutant was coexpressed with a quadruple domain mutant that has mutations in the other four catalytic domains, as follows; I: ACPm and KSmATmDHmKRm, II: KRm and KSmATmDHmACPm, III: DHm and KSmATmKRmACPm, IV: ATm and KSmDHmKRmACPm, V: KSm and ATmDHmKRmACPm. HPLC conditions were same as those used for Figure 3. All kinds of coexpression transformants could produce 6-MSA.

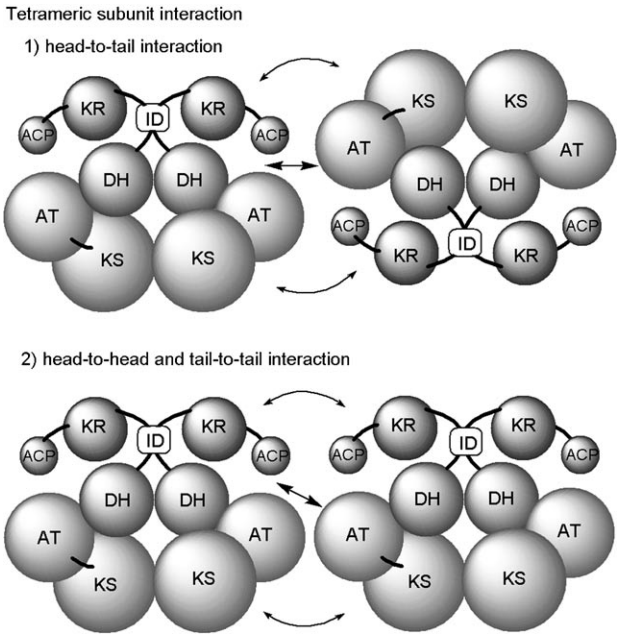


Figure 7. Proposed ATX tetramer model. "Head-to-head and tail-to-tail" subunit dimers might interact in a "head-to-head and tail-to-tail" or "head-to-tail" manner. Five catalytic domains could interact with each other with substantial flexibility in the reaction chamber.

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