Structure of the human β -ketoacyl [ACP] synthase from the mitochondrial type II fatty acid synthase

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Abstract

Two distinct ways of organizing fatty acid biosynthesis exist: the multifunctional type I fatty acid synthase (FAS) of mammals, fungi, and lower eukaryotes with activities residing on one or two polypeptides; and the dissociated type II FAS of prokaryotes, plastids, and mitochondria with individual activities encoded by discrete genes. The β -ketoacyl [ACP] synthase (KAS) moiety of the mitochondrial FAS (mtKAS) is targeted by the antibiotic cerulenin and possibly by the other antibiotics inhibiting prokaryotic KASes: thiolactomycin, platensimycin, and the α -methylene butyrolactone, C75. The high degree of structural similarity between mitochondrial and prokaryotic KASes complicates development of novel antibiotics targeting prokaryotic KAS without affecting KAS domains of cytoplasmic FAS. KASes catalyze the C₂ fatty acid elongation reaction using either a Cys-His-His or Cys-His-Asn catalytic triad. Three KASes with different substrate specificities participate in synthesis of the C₁₆ and C₁₈ products of prokaryotic FAS. By comparison, mtKAS carries out all elongation reactions in the mitochondria. We present the X-ray crystal structures of the Cys-His-His-containing human mtKAS and its hexanoyl complex plus the hexanoyl complex of the plant mtKAS from *Arabidopsis thaliana*. The structures explain (1) the bimodal (C₆ and C₁₀–C₁₂) substrate preferences leading to the C₈ lipoic acid precursor and long chains for the membranes, respectively, and (2) the low cerulenin sensitivity of the human enzyme; and (3) reveal two different potential acyl-binding-pocket extensions. Rearrangements taking place in the active site, including subtle changes in the water network, indicate a change in cooperativity of the active-site histidines upon primer binding.

Keywords: fatty acid synthesis; X-ray crystal structure; hexanoyl; acyl binding; antibiotics; binding pockets; cerulenin

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Abbreviations: ACP, acyl carrier protein; cerulenin, (2R,3S)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide; C75, tetrahydro-4-methylene-2-octyl-5-oxo-3-furancarboxylic acid; DTT, dithiothreitol; FAS, fatty acid synthase; KAS, β -ketoacyl [acyl carrier protein] synthase; mtKAS, mitochondrial KAS; RMSD, root-mean-square deviation; TCEP, tris(2-carboxyethyl)phosphine; thiolactomycin, 4-hydroxy-3,5-dimethyl-5-(2-methylbuta-1,3-dienyl)-5H-thiophen-2-one; TLS, translation/libration/screw; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Fatty acid biosynthesis was long thought to be differently organized in diverse organisms. The type I fatty acid synthase (FAS) complexes present in the cytoplasm of mammals, fungi, and lower eukaryotes are multifunctional proteins encoded by a single gene or, in some cases, two genes. Type II FAS present in prokaryotes and plant plastids consists of discrete enzymes. Prokaryotic fatty acid biosynthesis is being regarded as an obvious target for novel antibiotics (Campbell and Cronan 2001; Heath et al. 2001; Zhang et al. 2006b). Triclosan, which inhibits the enoyl reductase of type II FAS, is used in everyday applications such as toothpaste, sportswear, and kitchen utensils (Bhargava and Leonard 1996; Heath et al. 1998,

1999). The mammalian cytoplasmic type I FAS is the presumed target for the inhibitors cerulenin and C75, inducing apoptosis in some cancer cell lines and stopping weight gain in mice (Kuhajda et al. 2000; Loftus et al. 2000; Haber et al. 2003; Zhou et al. 2003; Alli et al. 2005). Initial evidence was obtained in the early 1990s for a mitochondrial type II FAS (Mikolajczyk and Brody 1990; Harington et al. 1993). These results were extended and have led to the recent cloning and characterization of the remaining mitochondrial FAS components from a range of organisms including yeast, humans, and *Arabidopsis thaliana* (Zhang et al. 2003; Kastaniotis et al. 2004; Yasuno et al. 2004), thus establishing the existence of a type II FAS in mitochondria. Its presence complicates the search for novel antibiotics as well as the interpretation of the effect of FAS inhibition in eukaryotic systems once thought to contain only the cytoplasmic FAS.

In prokaryotic FAS, the target of cerulenin, C75, thiolactomycin, and the recently reported inhibitor platenimycin is the condensing enzyme β -ketoacyl [acyl carrier protein] synthase (KAS) (D'Agnolo et al. 1973; Omura 1976; Nishida et al. 1986; Kauppinen et al. 1988; Kuhajda et al. 2000; Wang et al. 2006). KAS enzymes catalyze the Claisen condensation of a primer acyl chain with malonyl-acyl carrier protein (ACP) converting the primer to a β -ketoacyl-ACP elongated by a C_2 unit. The reaction mechanism is tripartite. (1) First, an ACP-activated primer acyl substrate accesses the buried active site through a funnel. Here it is *trans*-thioesterified to the active-site cysteine and the acyl chain inserted in the hydrophobic acyl-binding pocket. Then, (2) the donor malonyl-ACP accesses the active site through the same funnel entering the malonyl-binding pocket. Its decarboxylation generates a reactive C_2 -carbanion (3) that attacks and condenses with the α -carbon of the bound primer, resulting in its release as β -ketoacyl bound to ACP. The position of the active-site cysteine at the N-terminal end of an α -helix lowers its pK_a value, enabling *trans*-thioesterification. The decarboxylation reaction is accelerated through either primer binding, binding of inert substrates, or mutation of the active-site cysteine (Kresze et al. 1977b; Witkowski et al. 1999; McGuire et al. 2001). Three KAS isozymes with different substrate and inhibitor specificities are present in *Escherichia coli*—*EcKASI*, *EcKASII*, and *EcKASIII*. *EcKASIII* catalyzes the initial condensation reaction of acetyl-CoA and malonyl-ACP, while *EcKASI* and *EcKASII* both elongate saturated fatty acids up to C_{16} . *EcKASI* is essential for the initiation of unsaturated fatty acid synthesis (Garwin et al. 1980a), while *EcKASII* alone catalyzes the last elongation in unsaturated fatty acid biosynthesis, palmitoleic ($C_{16:1}$) to *cis*-vaccenic ($C_{18:1}$) acid (Rosenfeld et al. 1973; D'Agnolo et al. 1975; Garwin et al. 1980b). In the absence of acetyl-CoA, *EcKASI* decarboxylation

produces acetyl-ACP (McGuire et al. 2001), while little or none is detected using *EcKASII* and *Streptococcus pneumoniae* KASII (von Wettstein-Knowles et al. 2000; Zhang et al. 2006a). *EcKASI* is highly sensitive toward cerulenin, *EcKASII* moderately sensitive, and *EcKASIII* insensitive (D'Agnolo et al. 1975; Garwin et al. 1980a; Jackowski and Rock 1987; Edwards et al. 1997; McGuire et al. 2000). Human and yeast type I FASes are also sensitive to cerulenin, while thiolactomycin is FAS type II specific (Vance et al. 1972; D'Agnolo et al. 1973; Hayashi et al. 1983; Nishida et al. 1986; Jackowski et al. 1989). The three KAS isozymes constitute two groups; KASI + II have a Cys-His-His (CHH) catalytic triad, while KASIII has a Cys-His-Asn (CHN). The KAS activity of type I FASes and the mitochondrial KASes belongs to the CHH group. Crystallization of acyl-KAS complexes has failed to unveil the background of the different substrate specificities (Olsen et al. 2001).

The human mitochondrial KAS (*HsmtKAS*) was cloned and characterized by Zhang et al. (2005). Like the *A. thaliana* mitochondrial KAS (*AtmtKAS*) (Yasuno et al. 2004), *HsmtKAS* includes an N-terminal mitochondrial target peptide. *HsmtKAS* is less sensitive to cerulenin than other CHH enzymes and has a distinct bimodal substrate specificity (Zhang et al. 2005). To add to the understanding of KAS enzymes and to support the development of new antibiotics and anti-oncogenic FAS inhibitors specific for either mitochondrial or cytoplasmic KAS, we present the X-ray crystal structures of *HsmtKAS* and of the covalent hexanoyl complexes of *HsmtKAS* (*HsmtKAS:C₆*) and *AtmtKAS* (*AtmtKAS:C₆*). The structures shed light on rearrangements taking place in the active site upon substrate binding and on structural features important for inhibitor binding and substrate specificity differences between KASI and II enzymes.

Results

The structures of HsmtKAS and enzyme:acyl complexes

HsmtKAS was purified and its activity confirmed by decarboxylation assays (data not shown). Crystallographic analysis revealed that *HsmtKAS* is a dimer with the well-known α - β - α - β -thiolase fold first described by Mathieu et al. (1994) capped by an α -helical region connecting the strands of the N-terminal β -sheet (Fig. 1). Dimerization buries 3455 Å², similar to the case of *AtmtKAS* (Olsen et al. 2004), but 200–1200 Å² more than in the bacterial KAS enzymes. Whereas an extra α -helix in the cap region (Fig. 2B) contributed to the interface enlargement in *AtmtKAS*, an extra two-stranded N-terminal β -sheet and the burying of pockets that form the interface enlargement do so in *HsmtKAS*. The overall architecture is remarkably conserved. *AtmtKAS* and

A

	G78	I84	P93	F101	M154	L184	C209	D237	F248	H348	H385	F445	F447
<i>HsmtKAS</i>	VGEYK	IPC	YVPRGSDEGQFN	MG	I	KIVN	STACT	GCTDSC	AGFS	NAHATST	TGHL	NSFGFG	
<i>AtmtKAS</i>	TLDDLKMSFDEETKLYTFDQLSS		EVVPGSNPGEFD	GGIG	I	KIVN	VTACA	GCTESS	AGFS	NAHATST	TGHL	NSFGFG	
<i>ScmtKAS</i>	TSLPNYNEDYKLR	EK	TTTVGKIPEN	Q	SGIG	KIVN	STACA	GASETTS	AGFI	NAHATST	TGHL	NSFGFG	
<i>SpKASII</i>	TKFDHSD	FDV	ELQ	DFP	SGIG	KAMPN	NTACS	GCTEAS	AGFQ	NAHATST	TGHL	NSFGFG	
<i>SspKASII</i>	TRFDASD	QAC	EVK	DFD	TGIG	MMAN	VTACA	GCTEAA	AGFA	NAHATST	TGHL	NSFGFG	
<i>EcKASII</i>	DHFDTS	YAT	LVK	DFN	SGIG	STVNV	ATACT	GCAEKA	GGEF	NAHATST	TGHL	NSFGFG	
<i>EcKASI</i>	QELKDSG	MRS	NVK	LD	SGIG	KAS	SSACA	GCE	CEED	NSHGTST	TGHL	NSFGFG	
<i>HsFAS</i>	DRRWKAG	LYGLP	KKDLS	RFD	VSGS	GCQRA	DTACS	GGINVL	VOEL	EAHGTCT	MGHPEP	NSFGFG	
					aa	a	aa	a	a		a	a	
					ccc	ccc	c						

B

Figure 1. (A) Alignment of relevant sequence segments of KAS enzymes, numbered as in *HsmtKAS*. Residues bordering the *HsmtKAS* acyl-binding pocket are marked with the letter “a,” while those bordering the connecting tunnel are marked with the letter “c.” Shading refers to the degree of conservation. The sequences are *AtmtKAS* (Yasuno et al. 2004), *EcKASI* (Kauppinen et al. 1988), *EcKASII* (Edwards et al. 1997), *Saccharomyces cerevisiae* mtKAS (*ScmtKAS*) (Harrington et al. 1993), *S. pneumoniae* KASII (*SpKASII*) (Price et al. 2003), *Synechocystis* sp. KASII (*SspKASII*) (Moche et al. 2001), and the human KAS domain of type I FAS (*HsFAS*) (Jayakumar et al. 1995). (B) Cartoon representation of *HsmtKAS*:C₆ dimer in stereoview. The acyl chain is shown in orange spheres and selected residues in red sticks: the active-site residues Cys209, His348, His385, Phe447, and the switch Met154. One subunit is colored light gray and labeled with blue amino acid residue numbers, the other is colored according to the structural regions of the protein. The N- and C-terminal regions are blue and yellow, respectively, except for the α -helical extrusions forming the cap region, which are green. The two NH₄⁺ ions (cyan) present below the active sites are represented as spheres. The illustration was prepared in PyMol (DeLano 2002).

EcKASII are the closest structural neighbors to *HsmtKAS* (normalized RMSD₁₀₀ = 0.6 Å for 399/394 C_α positions) (Carugo and Pongor 2001); *EcKASI* comes next (0.8 Å, 380 C_α), and finally *EcKASIII* (1.27 Å, 198 C_α). A loop (Pro93–Phe101) connecting the first and fourth strands of the N-terminal β -sheet is unique to the mitochondrial KAS enzymes (Fig. 2A). Despite the structural similarity, a significant difference is the lack of the α -helical insertion characterizing *AtmtKAS*, which is replaced by a loop, Gly78–Ile84, resembling that of the KASI and II enzymes (Fig. 2A).

The crystal structures of the hexanoyl complexes do not deviate significantly from the structures of the free enzymes (RMSD = 0.5 and 0.3 Å between 3023 *HsmtKAS* and 3222 *AtmtKAS* atoms, respectively). The major deviations between *HsmtKAS* and *HsmtKAS*:C₆ are located in the cap region as illustrated by the tube diameters of the C_α RMSD comparison of Figure 2A and

the green color in the secondary structure bar indicator in Figure 2C. Apart from the extreme RMSD value in the 424–427 loop at the surface of subunit B, little structural change is seen in the *AtmtKAS*–*AtmtKAS*:C₆ comparison (Fig. 2B,D); nevertheless, also in this case the largest deviations are found in the cap region. The overall lower RMSD level observed between the *AtmtKAS* structures compared to the *HsmtKAS* structures might be a result of differences in crystal packing interactions.

Acyl-binding-induced rearrangements in the active site

The active-site catalytic CHH triad is located within a 5 Å radius at the bottom of a 15 Å deep water-lined funnel with a molecular volume of 1260 Å³ (Fig. 3A, red balls and sticks close to purple surface). [All volumes given in the text are molecular (Connolly's) volumes calculated with CASTp (Binkowski et al. 2003).] The overall

Figure 2. C_{α} RMSDs between (A) *HsmtKAS* and *HsmtKAS:C₆* and (B) *AmtKAS* and *AmtKAS:C₆*. The tube diameter is proportional to the RMSD between the structures. The extreme RMSD values are 0.01 and 1.66 Å in *HsmtKAS-HsmtKAS:C₆* and 0.01 and 2.32 in *AmtKAS-AmtKAS:C₆*. The colors, sticks, and spheres are as in Figure 1. (C,D) Plots of the C_{α} RMSDs of *HsmtKAS* and *AmtKAS* subunits A and B, respectively. Schematic drawings of the secondary structural elements bar with color coding as in Figure 1 are inserted underneath the plots.

architecture of the active site resembles other characterized CHH enzyme structures (Fig. 4). Upon formation of the acyl-enzyme complex, the active site subtly rearranges. In the complex the C_{ζ} of Phe447 moves 1.25 Å relative to its position in the *HsmtKAS* structure (Figs. 3B, 4A), and the phenyl residue is fixed with the plane of the aromatic ring parallel to the bound acyl chain, 3.7 Å from the ligand C2 atom (Figs. 3C, 4A). The Phe447

B-factors in the complex decrease by ~50% to the level of the surrounding residues.

In *HsmtKAS*, a high B-factor water molecule, W1, is located above the active-site Cys209 (Fig. 4A, center). This particular water molecule is conserved throughout known KAS structures, and under scrutiny for playing a role in decarboxylation as a nucleophile activated by His348 (Zhang et al. 2006a). In *HsmtKAS*, W1 is within

Figure 3. Cavities close to the Met154 switch in the acyl-binding pocket of *HsmtKAS*. (A) Overall view of the cavities present in *HsmtKAS*. The active-site access funnel is in purple, the acyl-binding pocket plus active-site residues are in red, Met154 is in cyan, the water-free extension plus Phe248 is in green, and the water-filled connecting tunnel joining the Met154s plus Leu184 is in blue. The backbone traces of subunits A and B are included in cartoon representation in light and dark gray. (B,C) Close ups of the cavities in *HsmtKAS* and *HsmtKAS*:C₆, respectively. (D) A modeling of *HsmtKAS*:C₆ with Met154 shown in a rotamer allowing access to the water-free extension. (E) The acyl-binding pocket of *EcKAS1*:cerulenin complex (PDB code: 1FJ8) (Price et al. 2001a). The surfaces are colored as above. Cerulenin is included in balls and sticks inside the acyl-binding pocket. The catalytic triad is included in red balls and sticks, and Met197 occupies the water-free extension; Gln113 and Glu200 are in gray sticks. All surfaces and cavities were calculated with a 1.3 Å probe in MSMS (Sanner et al. 1996), and the illustration was prepared with Dino3D (Philippsen 2002) and Povray. (F) Cartoon showing the acyl-binding pocket rearrangements necessary for long chain substrate binding.

Figure 4. Stereoviews of the *HsmtKAS* active site. (A) C₆ in the acyl-binding pocket. Balls and sticks are colored according to atom type and origin: C atoms from *HsmtKAS* and the *HsmtKAS*:C₆ are green and light blue, respectively; N atoms, dark blue; O atoms, red; and S atoms, yellow. Selected water molecules are included as red (*HsmtKAS*) and blue (*HsmtKAS*:C₆) spheres. The $F_o - F_c$ map, calculated excluding the C₆ atoms from the model, is contoured at 3σ . Atoms within hydrogen bond distance of each other are connected with dotted lines, colored blue in the *HsmtKAS* structure and green in the complex. The shortest distance between the ligand and Met154 is included in black. (B) Model of cerulenin from the *EcKASII*:cerulenin complex (PDB code: 1B3N) (Moche et al. 1999) (red sticks) and the *EcKASI*:cerulenin complex (PDB code: 1FJ8) (Price et al. 2001a) (orange sticks) made by superimposing the cerulenin containing structures on the *HsmtKAS*:C₆ structure. The illustration was prepared in PyMOL.

hydrogen bond distance of Phe445 O and His348 N_ε (Fig. 4A, center left, dashed green lines). In the *HsmtKAS*:C₆ complex, W1 is displaced 0.7 Å toward His385 N_ε entering hydrogen bond distance of this atom and breaking the hydrogen bond with the backbone O of Phe445 (Fig. 4A, dashed blue lines). The W1 B-factor drops to the level of the surroundings, and a network of ordered water molecules appears above the active site. One of these water molecules bridges the broken bond between Phe445 and W1 (Fig. 4A, center left, blue sphere). Another water molecule, W2, situated on the other side

of His348 moves into hydrogen bond distance of the N_ε of this residue (Fig. 4A, lower center, blue sphere and dashed blue line).

In the *AtmtKAS*:C₆ complex, significant side-chain rearrangements in the active site are not observed, and only small adjustments in the active-site water structure follow formation of the thioester bond. Whereas W2 movement is different in *AtmtKAS*:C₆ and the *E. coli* KASI and II complexes, the adjustments in the W1 position are consistent with the water structure adjustment mentioned above (Olsen et al. 2001; von Wettstein-Knowles et al.

2006). The B-factors of the *AtmtKAS* Phe447 analog shift likewise.

A switch in the acyl-binding pocket

In *HsmtKAS*:C₆ and *AtmtKAS*:C₆, electron densities extending from the active-site Cys209s into the cap were modeled as hexanoyl *trans*-thioesterized to the active-site Cys (Fig. 4A, $F_o - F_c$ omit map, gray mesh). In the uncomplexed forms buried, hydrophobic-residue-lined cavities are present from Cys209 to Met154 in *HsmtKAS* and to Ile154 in *AtmtKAS* (Met/Ile154) (Fig. 1A; Fig. 3A,B, acyl binding pocket, red; Fig. 4B; Table 1). They are 10 Å long with molecular volumes of ~115 Å³.

The cavities are not solvent accessible prior to ligand binding because Phe447 blocks the entrance (Fig. 3A,B). Asp/Glu237 is situated at the end of the acyl-binding pockets, but does not contribute to the pockets' hydrophilicity as the carboxyl O is hydrogen bonded to the backbone Ns of Gly388 and Gly153 (not included in the figure). In the C₆ complexes, the acyl chains fill the pocket causing minimal distortion (Figs. 3C, 4A). A maximum of one extra C atom is allowed in the pocket without reorientation of Met/Ile154. Separate from the acyl-binding pocket immediately beyond Met/Ile154, two as yet undescribed cavities exist in *HsmtKAS* and *AtmtKAS* (Fig. 3A,B). (1) Met/Ile154 and Leu184 restrict access to a water-lined cavity crossing the dimer interface and connecting to Met/Ile154 of the other subunit (Figs. 3A,B, connecting tunnel, blue). (2) Met/Ile154 and a small water-free hydrophobic cavity (Figs. 3A,B, water-free extension, green) exist immediately above and behind Met/Ile154 and Phe248 (Figs. 3A,B, 4B). Upon C₆ ligand binding, the same cavities exist, except that the water-free extension is only found in one of the *HsmtKAS*:C₆ subunits because of a difference in Met154 orientation between the subunits. The orientation of the neighboring Cys239 residue also differs between the subunits of *HsmtKAS*, indicating flexibility in the region (Fig. 3B,C). A scanning of optimal rotamers of Met154 in *HsmtKAS*:C₆ and Ile154 in *AtmtKAS*:C₆

shows that the acyl-binding pocket can be extended into the water-free extension by a rotation in the Met/Ile154 χ -angles enlarging the pocket to a total of 220 Å³ (Figs. 3D, red surface). In addition to the water-free extension, the increased volume represents the volume previously occupied by Met154 and Phe447. The extended acyl-binding pocket has room for C₁₂ substrates. None of the Met/Ile154 rotamers in either *HsmtKAS* or *AtmtKAS* allowed access from the acyl-binding pocket to the connecting tunnel. On the basis of these characteristics, we postulate that Met/Ile154 functions as a switch.

Discussion

The framework for the decarboxylation reaction

Acyl chain elongation by the Claisen condensation reaction mechanism includes generation of a carbanion from malonyl by decarboxylation (von Wettstein-Knowles et al. 2000). That His385 promotes decarboxylation by hydrogen bond donation from N_ε is well established (Olsen et al. 2001; von Wettstein-Knowles et al. 2006; Zhang et al. 2006a). The electronic state of His348 and its part in decarboxylation remain enigmatic. Upon acylation of the active-site cysteinyl, the side chain of Phe447 in *HsmtKAS* changes from poorly defined to a fixed rotamer, and W1 is fixed within hydrogen bond distance of both histidine N_εs. The rearrangements taking place indicate a possible change in the state of His348. Primer binding to the active-site cysteinyl accelerates the decarboxylation reaction, even with inert primers such as iodoacetamide (Kresze et al. 1977a). The same is true when the active-site cysteinyl has been mutated to Gln or Ala (Witkowski et al. 1999; McGuire et al. 2001), suggesting that the change most likely originates in the active-site pocket propagating through the imidazole ring of His348. The positional adjustment and fixation of W1 directly connected to the active-site histidines in the KAS:acyl complexes reflect the rearrangement activating decarboxylation upon primer binding. The positional adjustment may result from the conversion of the hydrophilic

Table 1. Amino acid composition and lengths of intramolecular pockets in *HsmtKAS*

	Subunit	Hydrophilic residues	Hydrophobic residues	Length (Å)
Acyl-binding pocket	A	D237 ^A	G153 ^A , M154 ^A , A208 ^A , C209 ^A , F248 ^A , L387 ^A , G388 ^A , F447 ^A , L184 ^B	10 ^a
	B	D237 ^B	G153 ^B , M154 ^B , A208 ^B , C209 ^B , C239 ^B , F248 ^B , L387 ^B , G388 ^B , F447 ^B , L184 ^A	10 ^a
Water-free extension	A	S244 ^A	M154 ^A , L157 ^A , F248 ^A , F179 ^B , V180 ^B , I183 ^B	6.5 ^b
	B	S244 ^B	M154 ^B , L157 ^B , G247 ^B , F248 ^B , F179 ^A , V180 ^A , I183 ^A	6.5 ^b
Connecting tunnel	A–B	S206	G153, M154, I155, I183, L184, V185	10 ^c

Superscripts A and B designate the subunit of the residue.

^aCys209–Met154 distance.

^bMet154–Phe179 distance.

^cMet154^A–Met154^B distance.

active-site cysteinyl to a hydrophobic thioester, prompting a fixed network of structured water molecules to appear. Thus, when poised for decarboxylation, W1 is positioned immediately between the active site histidines, suggesting that both N_εs interact with the same substrate atom, unlike the various mechanisms proposed by Olsen et al. (2001), Witkowski et al. (2002), and Zhang et al. (2006a). The accelerated production of the derailment product triacetic acid lactone (TAL) in *S. pneumoniae* KASII Phe447Ala, shows that Phe447 is not required for decarboxylation, as more substrate is being consumed in its absence. Instead, a role for Phe447 as inhibitor of decarboxylation in the situation where no primer is bound is more compatible with the results of Zhang et al. (2006a).

Acylation and its implications for inhibitor binding

With a molecular volume of $\sim 115 \text{ \AA}^3$ and a depth of 10 $\text{\AA}$, the longest acyl chain fitting in the binding pocket without distorting Met154 is C₇. This is far below the apparent maximum *HsmtKAS* substrate length of C₁₄ (Zhang et al. 2005) and too little for binding of the C₁₂ antibiotic cerulenin (Fig. 4B, orange and red, balls and sticks). In *EcKASII*, the pocket is equally small. The Met154 homolog Ile108 terminates the binding pocket, giving a volume of 71 $\text{\AA}^3$ (Huang et al. 1998). The crystal structure of the cerulenin–*EcKASII* complex reveals that a rearrangement of Ile108 takes place, expanding the pocket to 215 $\text{\AA}^3$, which is comparable to the modeled 220 $\text{\AA}^3$ extended acyl-binding pocket in *HsmtKAS*:C₆ (Fig. 3D, red acyl-binding pocket; Moche et al. 1999). That the region is likely to harbor such rearrangements in *HsmtKAS* is indicated by torsion/libration/screw (TLS) analysis (Painter and Merritt 2006). TLS analysis allows the description of anisotropic, static disorder on a per segment basis. Using 20 segments, TLS analysis of *HsmtKAS* and *HsmtKAS*:C₆ showed that in *HsmtKAS*:C₆, the segments surrounding the hypothesized pocket extension add flexibility to this volume. The major changes taking place upon binding the C₆ ligand in *HsmtKAS* localize to this part of the structure as well (Fig. 2A). Likely this extended pocket will be available for binding cerulenin and medium to long chain acyl substrates in *HsmtKAS* and *AtmtKAS* (Fig. 3F).

The energy barrier caused by the required rearrangement of Met154 explains the low C₈-substrate preference ($K_{\text{cat}}/K_{\text{m}}$) observed by Zhang et al. (2005). With respect to the longer C₁₀ and C₁₂ substrates, the cost of rearranging Met154 is compensated by interaction with the highly hydrophobic hypothesized extension. Binding of substrates longer than C₁₂, which have very low $K_{\text{cat}}/K_{\text{m}}$ values (Zhang et al. 2005), is presumably impeded by their need to expand the pocket further. The higher

sensitivity toward cerulenin of *AtmtKAS* (Olsen et al. 2004) than *HsmtKAS* (Zhang et al. 2005) may reflect the linear character of Met versus Ile. That the position occupied by Met154 plays a central role in substrate binding was first highlighted by a mutation study (Val et al. 2000), where Ile108Phe in *EcKASII* exhibited a dramatic reduction in elongation activity with substrates longer than C₆.

Analysis of ligand-free and longer acyl chain ligand–KAS complexes shows that in *EcKASI* complexes [C₁₂: PDB no. 1EK4 (Olsen et al. 2001); cerulenin: 1FJ8 (Price et al. 2001b)], the acyl-binding pocket extends toward the connecting tunnel (Figs. 3E, 4B), while in the KASII–cerulenin complex, the ligand extends toward the possible pocket extension identified in this study (Figs. 3F, 4B). In the KASI structure, a methionine positioned in the cap region, *EcKASI* Met197, occupies the water-free pocket extension, making it unavailable for substrate binding. KASII structures have no access to a pocket for long chain substrates resembling the part of the KASI acyl-binding pocket harboring the ω -end of long chain fatty acids. The combined action of Met154, Ile183, and Leu184 effectively obstructs the formation of a similar binding pocket in *HsmtKAS*. The active sites of the two subunits are not connected in available KASI structures, since the side chains of *EcKASI* Gln113 and Glu200 interact at the dimer interface (Fig. 3E). These observations indicate substantial differences between the acyl-binding pockets of KASI and KASII enzymes providing an explanation for the variations in substrate specificity between KAS I and II enzymes.

Superposition of the structures of *HsmtKAS* and the *EcKASII*–platensimycin complex (Wang et al. 2006) reveals that *HsmtKAS* should provide as good a target for platensimycin as *EcKASII*. In *HsmtKAS*:C₆, Phe447 adopts the conformation allowing edge-to-face aromatic stacking suggested as central for platensimycin binding (Wang et al. 2006). Arg252 positioned at the beginning of the active-site access funnel is the sole potential impediment for ketolide binding. As Arg252 is disordered in *HsmtKAS* and found in two alternative conformations in *HsmtKAS*:C₆, this is unlikely to cause problems. The malonyl-binding pocket, as defined in the *EcKASI*:thio-lactomycin complex (Price et al. 2001a), is conserved in *HsmtKAS* (Pro317, Phe445, and Phe447).

The low degree of similarity between the residues lining the acyl-binding pocket in *HsmtKAS* and the human KAS domain of FAS (Fig. 1A) suggests that acyl tail modifications of cerulenin might provide potential targeting differences between *HsmtKAS* and human type I FAS. Tetrahydrocerulenin lacking double bonds in the acyl tail does not affect human type I FAS; nevertheless, it inhibits T24 cell proliferation (Lawrence et al. 1999). Tetrahydrocerulenin is active against *EcKASII*

(D'Agnolo et al. 1973; Moche et al. 1999) and fungal type I FAS (Morisaki et al. 1993). Interestingly, the primary structure of the KAS domain of human type I FAS diverges from the KASII sequences in the region around M154, where all prokaryotic KASIIs have Ile and *EcKASI* has a Gly (Fig. 1A).

Conclusion

Likely, the state of the active-site histidines of KASI + II enzymes changes upon substrate binding. When binding acyl chains longer than C₇, the acyl-binding pockets of *HsmtKAS* and *AtmtKAS* could be extended through a change in the rotamer of the Met/Ile154 switch residues. The rotation will extend the pockets into cavities similar to the one made available in *EcKASII* upon cerulenin binding. In *EcKASI*, this pocket is blocked by Met197, and long chain acyl substrates are passed toward a pocket similar to the connecting tunnel. The different potential pocket extensions are described here for the first time, and their presence offers an explanation for the different substrate specificities observed in KASI+II enzymes.

The coordinates and structure factors have been deposited in the Protein Data Bank at EBI (<http://www.ebi.ac.uk/msd>) (IDs: *HsmtKAS*, 2IWY; *HsmtKAS*:C₆, 2IWZ; *AtmtKAS*:C₆, 2IX4).

Materials and methods

Identification of the *HsmtKAS*

To identify a putative *HsmtKAS*, the expressed sequence tag (EST) database was searched with the sequence of *EcKASII* (Siggaard-Andersen et al. 1994). The sequence with the highest degree of similarity (GenBank accession no. AK000611.1) encoded a putative KAS. The primary sequence translation product was 51% identical to *AtmtKAS*, 46% identical to the *EcKASII* sequence, and 35% identical to *EcKASI* sequence. The catalytic CHH triad and residues assigned a role in the structure or reaction mechanism of KASI/KASII were all conserved in the sequence including the *EcKASI* Lys328 and Phe395. The primary sequence included a 38 amino acid N-terminal target sequence, predicted to target mitochondria by TargetP 1.0 (Emanuelsson et al. 2000).

Cloning, expression, and protein purification

A clone of AK000611 was kindly provided by Sumio Sugano, Tokyo University (Yudate et al. 2001). Using the forward primer 5'-GGTGGTGGTGGATCCATTGAGGGGCGCTCCAGATTG CATAGGC-3' (having a BamHI restriction site in bold and coding for a factor Xa cleavage site) and the reverse primer 5'-GGTGGTGGTAAGCTTCTACAGTCCAGCAAT-3' (HindIII site in bold), the coding sequence was ligated into pQE-30 (QIAGEN), substituting the 37-codon leader with an MRGS-HHHHHHGS-IEGR tag. The resultant plasmid was transformed into the *E. coli* XL-1 blue strain (Stratagene), verified by

sequencing, and transformed into the *E. coli* expression strain M15. Protein was expressed and purified as previously described (McGuire et al. 2001), except that DTT was substituted with the more stable TCEP (0.2 mM), sonication was for five instead of three rounds, and induction was overnight at 20°C. The protein was further purified by anion exchange chromatography on a MonoQ HR 10/10 column (Amersham Biosciences) mounted on an ÄKTA Purifier HPLC system (Amersham Biosciences) with a pH 9 Tris-HCl buffer system [A buffer: 30 mM Tris at pH 9, 10 mM NaCl, 0.2 mM EDTA, 0.2 mM TCEP, and 8.7% (w/v) glycerol; B buffer: buffer A + 2 M NaCl]. After elution, the buffer was changed to storage buffer [150 mM NaCl, 30 mM Tris at pH 7.8, 2 mM EDTA, 0.2 mM TCEP 8.7% (w/v) glycerol], and the protein was concentrated to 4 mg/mL in an Amicon Ultra-15 filter device (Millipore) and stored at -20°C. Decarboxylation activity was verified in conformationally sensitive urea gels as carried out by McGuire et al. (2001). *AtmtKAS* was produced as described previously (Olsen et al. 2004).

Crystallization and data collection

Crystals of *HsmtKAS* and *HsmtKAS*:C₆ were grown in hanging drops. Drops were composed of 2 µL of 87 µM protein solution in storage buffer and 2 µL of reservoir solution containing 24% (w/v) polyethylenglycol 3350 and 0.2 M NH₄Cl. In the co-crystallization experiment, 4 mM hexanoyl-CoA was added to the protein solution prior to setting up the drops. After 5–8 d at room temperature, rod-shaped single crystals appeared. The crystals were flash cooled in N_{2(l)}. Data were collected at 100 K to 2 Å from the *HsmtKAS* crystal at beamline I711 (Cerenius et al. 2000), MAXlab, Lund University; and to 1.6 Å from the complex at beamline X11 at EMBL, Hamburg. High- and a low-resolution (3.5 Å cutoff) oscillation scans were collected from the *HsmtKAS*:C₆ complex. The exposure time was halved, and the oscillation angle per frame was increased from 0.2° to 0.5° for the low-resolution scan. Data were integrated with MOSFLM and merged and scaled with SCALA (Evans 1997).

Attempts to soak acyl-CoA derivatives into the previously characterized *AtmtKAS* crystals (Olsen et al. 2004) failed. Screening of co-crystallization conditions resulted in plate-shaped single crystals when hanging drops were incubated at 22°C. Two microliters of a protein solution (5 mg/mL *AtmtKAS*, 20 mM bis-tris-buffer at pH 6.0, 200 mM KCl, 2 mM DTT) was mixed with 2 µL of 0.1 M MES (pH 6.5), 12% (w/v) PEG20000, and 1 µL of 50 mg/mL hexanoyl-CoA. The crystals were immersed in 35% (w/v) PEG4000, 0.1 M bis-tris (pH 6.5) for 5 sec and flash cooled in N_{2(l)}. Diffraction data to 1.95 Å resolution were collected at the X11 beamline, EMBL Hamburg.

Phasing and model building

The phases of the *HsmtKAS* structure factors were assigned by molecular replacement using the structure of *AtmtKAS* without water molecules (Olsen et al. 2004), as search model with MOLREP (Vagin and Teplyakov 1997). The model was built automatically using ArpWarp (Perrakis et al. 1999) with one homodimer in the asymmetric unit. The model was improved by hand building in O (Jones et al. 1991) and Coot (Emsley and Cowtan 2004), supplemented first by rigid-body refinement and simulated annealing in CNS (Brünger et al. 1998) and at later stages by positional refinement in REFMAC5 (Collaborative Computational Project, No.4 1994; Murshudov et al. 1997). Water molecules were added using the water picking procedure

Table 2. Data collection, phasing, and refinement statistics

	<i>HsmtKAS</i>	<i>HsmtKAS:C₆</i>	<i>AtmtKAS:C₆</i>
Data collection			
Beam line	MAXLAB/I711	DESY/X11	DESY/X11
Wavelength (Å)	1.063	0.8157	0.8157
Temperature (K)	100	100	100
Crystal class	Orthorhombic	Orthorhombic	Monoclinic
Spacegroup	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁
Cell dimensions (Å)	$a = 72.6; b = 96.1; c = 115.5$	$a = 71.9; b = 95.9; c = 114.6$	$a = 72.1; b = 92.9; c = 74.2; \beta = 106.6$
Resolution range (Å)	36.9–2.05 (2.15–2.05) ^a	32.3–1.65 (1.74–1.65) ^a	71.1–1.95 (2.05–1.95) ^a
Number of unique reflections	51,373	95,846	67,193
R_{merge} (%) ^b	7.5 (27.3) ^a	8.5 (37.4) ^a	11.3 (37.9) ^a
Multiplicity	4.3	5.8	3.7
Completeness (%)	98.1	99.9	95.6
Average $I/\sigma I$	6.8 (2.7) ^a	6.0 (2.0) ^a	4.8 (1.8) ^a
Refinement statistics			
Number of reflections	47,479	90,951	64,327
Resolution range (Å)	73.92–2.05 (2.11–2.05) ^a	73.92–1.65 (1.69–1.65) ^a	71.1–1.95 (2.00–1.95) ^a
Number of residues	843	852	861
Number of ions	2	2	2
Number of water molecules	415	675	489
R -factor (%) ^c	16.1	15.5	19.5
R_{free} (%) ^d	20.5	18.8	22.4
Mean B-factor (Å ²)	28.7	25.0	23.5
Ramachandran plot (%) ^e			
Most favored	90.9	90.7	90.3
Additionally allowed	7.5	7.3	8.4
Generously allowed	1.0	1.3	0.5
Disallowed	0.6	0.7	0.7
RMSD			
Bond lengths (Å)	0.009	0.010	0.006
Bond angles (°)	1.103	1.277	0.934
Diffraction data precision indicator, DPI (Å) ^f	0.19	0.09	0.13

^aLast shell.^b $R_{\text{merge}} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, calculated in SCALA.^c R -factor = $\sum_h ||F_o| - k|F_c|| / \sum_h |F_o|$, calculated in REFMAC.^d R_{free} calculated as R -factor, but from 5% of the observations that had been excluded from the refinement.^eCalculated with PROCHECK (Laskowski et al. 1993).^fDiffraction data precision indicator, $DPI = \sqrt{N_{\text{atoms}}/N_{\text{obs}}} \times C^{-1/3} \times d_{\text{min}} \times R_{\text{free}}$, where N_{atoms} is the number of atoms in the model, N_{obs} the number of observed reflections, C the fractional completeness, and d_{min} the maximum resolution. Calculated in REFMAC according to Cruickshank (Cruickshank 1999).

in CNS. One solvent molecule in each subunit's active site was refined as an NH_4^+ ion. The phases from the refined *HsmtKAS* model were used to solve the structure of the covalent *HsmtKAS:C₆* complex, which was refined as above except that a hexanoyl molecule was fitted into an excess electron density emerging from the active-site thiol. Water was included with Coot, and the Babinet method was used to model bulk solvent. To fully exploit the quality of the data sets, TLS anisotropic refinement was applied using tensors generated with the online TLSMD server (Schomaker and Trueblood 1968; Painter and Merritt 2006). The *HsmtKAS* structure was refined with five segments per subunit (residues A36–A158, A159–A284, A285–A320, A321–A431, A432–A459, B37–B130, B131–B239, B240–B312, B313–B430, B431–B459), and the complex with two segments per subunit (A30–A180, A181–A459, B35–B158, B159–B459). Only the last four residues of the engineered

leader sequence were ordered and only in the A molecule of the *HsmtKAS* structure. In the B molecule, only the backbone and C $_{\beta}$ atom of the last residue of the engineered sequence could be traced. The orientation of a few residues and side chains could not be determined and were either left out (–) or included in truncated form—*HsmtKAS*: A (Arg252 C $_{\alpha}$, Thr315–, Ala316–, Pro317–, Glu322 C $_{\beta}$, Thr434–, Glu435–, Lys436–); *HsmtKAS:C₆*: A (Glu98 C $_{\beta}$, Lys433 C $_{\beta}$, Thr434 C $_{\alpha}$, Glu435 C $_{\alpha}$, Lys436 C $_{\alpha}$), B (Glu320 C $_{\beta}$). The following residues occurred in alternative conformations: *HsmtKAS* A (Val337, Gln338); *HsmtKAS:C₆* A (Gln104, Lys109, Met130, Lys197, Ser244, Arg252, Val337), B (Arg39, Ser96, His365).

The structure of *AtmtKAS* could be solved by rigid-body refinement in REFMAC5, giving an asymmetric unit of one homodimer. The structure of the *AtmtKAS:C₆* complex was refined using REFMAC5 and Coot by first, manually rebuilding

the protein main and side chains in Coot; second, including water molecules from the Coot interface; third, including ligand molecules from the Coot interface; and last, modeling the two cations identified in the structure. No NCS symmetry restraints were applied in the refinement. Models were refined with NH_4^+ ions, Mg^{2+} ions, Ca^{2+} , and K^+ ions with occupancy of 1. Only the K^+ and Ca^{2+} ions gave satisfactory B factors with these settings, and ligand–cation distances (average O–cation distance = 2.8 Å) are in accordance with the final interpretation of the ions as K^+ (Harding 2006). The side chain of Thr354 has been modeled in two alternate conformations. One TLS segment was defined for each subunit. TLS group parameters were refined in REFMAC5 after resetting the B factors to 20. The inclusion of TLS resulted only in a limited drop in the R_{free} (0.5%).

The data collection and refinement statistics are given in Table 2.

Acknowledgments

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