The thioesterase FlK from the fluoroacetate-producing Strep
tomyces cattleya catalyzes the hydrolysis of fluoroacetyl-coen
zyme A. This provides an effective self-defense mechanism, pre
venting any fluoroacetyl-coenzyme A formed from being
further metabolized to 4-hydroxy-trans-aconitate, a lethal
inhibitor of the tricarboxylic acid cycle. Remarkably, FlK does
not accept acetyl-coenzyme A as a substrate. Crystal structure
analysis shows that FlK forms a dimer, in which each subunit
adopts a hot dog fold as observed for type II thioesterases.
Unlike other type II thioesterases, which invariably utilize either
an aspartate or a glutamate as catalytic base, we show by site-
directed mutagenesis and crystallography that FlK employs a
catalytic triad composed of Thr42, His76, and a water molecule,
alogous to the Ser/Cys-His-acid triad of type I thioesterases.
Structural comparison of FlK complexed with various substrate
analogues suggests that the interaction between the fluorine of
the substrate and the side chain of Arg120 located opposite to the
catalytic triad is essential for correct coordination of the sub-
strate at the active site and therefore accounts for the substrate
specificity.

In 1986, Sanada et al. (1) discovered that Streptomyces cat-
tleya is able to produce fluorooacetate (FAc)8 from fluoroxide.
Attempts to elucidate the mechanism of this unusual transfor-
mation have led to the discovery of a fluorinase enzyme cata-
lyzing the formation of 5'-fluoro-5'-deoxyadenosine from
S-adenosyl-l-methionine and fluoride (2–6). We have cloned
the fluorinase gene flA (5) and subsequently identified a gene
cluster (fl) surrounding the fluorinase gene from S. cattleya (7).
Characterization of the 5'-fluoro-5'-deoxyadenosine phos-
phorylase (3), the enzyme responsible for the second step of
the FAc biosynthesis encoded by the flB gene located next to the
flA, has confirmed the involvement of the fl gene cluster in the
biosynthesis of FAc (7). In our efforts to further elucidate the
functions of the fl gene cluster, the protein encoded by the
flK gene located close to the 3'-end of the cluster was charac-
terized as a thioesterase that cleaves fluoroacetyl-coenzyme A
(FAcCoA) to produce FAc and CoA. It is noteworthy that
acetyl-coenzyme A (AcCoA) is not hydrolyzed by FlK (7).
Previous studies have shown that FAc can be used by AcCoA syn-
thase to synthesize FAcCoA, and citrate synthase can catalyze
formation of 2-fluorocitrate from FAcCoA and oxaloacetate (8).
Lauble et al. (9) have shown that the (−)-erythro diaste-
reomer of 2-fluorocitrate is further converted to 4-hydroxy-trans-
aconitate, a lethal competitive inhibitor of aconitate, thereby
blocking the tricarboxylic acid cycle. The presence of a
FAcCoA-hydrolyzing enzyme that has no effect on the meta-
bolically important intermediate AcCoA may therefore serve as
a self-resistance mechanism for the FAc-producing S. cattleya.

FlK is homologous to many predicted thioesterases and
hypothetical proteins according to protein-protein BLAST
searches against a non-redundant protein sequence data base
(7). However, none of these putative homologues has a defined
function. Two groups of thioesterases have been identified,
thioesterases I and thioesterases II, based on differences in their
amino acid sequence, protein folds, and catalytic mechanisms.
Type I thioesterases have evolved within the
thioesterases I and thioesterases II, based on differences in their
hypothetical proteins according to protein-protein BLAST
searches against a non-redundant protein sequence data base
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Type I thioesterases have evolved within the
thioesterases I and thioesterases II, based on differences in their
amino acid sequence, protein folds, and catalytic mechanisms.
Crystal Structure of Fluoroacetyl-CoA Thioesterase

is therefore expected to have a carboxylic side chain at the active site. Protein sequence alignment analysis has revealed that nine amino acid residues in FlK (Gly8, Thr42, Glu50, Leu61, Gly69, His76, Ala78, Gly83, and Gly116) are all conserved in AcCoA.

strate specificity of FlK for FAcCoA over the closely related and without bound analogues of FAcCoA. Our studies provide tallography of the wild type and mutant FlK proteins both with putative catalytic residues and defined structures by x-ray crys-

knowledge, no functionally characterized thioesterase has been

together with Glu50 among the conserved residues led us to speculate that FlK may employ a variation of the classical cata-

lytic triad-type mechanism with Thr42 (instead of a Ser or a Cys), His76, and Glu50 (instead of an Asp). His76 would then serve as a base to deprotonate Thr42. The deprotonated Thr42-

Oγ is proposed to act as a nucleophile attacking the fluoro-

cetyl carbonyl carbon of FAcCoA to initiate the hydrolysis reaction and the carboxylic side chain of Glu50 to neutralize the charge developed on the His76 side chain during the transition state of the reaction by hydrogen bonding with the imidazole nitrogen of His76 (7). Although a hydroxyl derived from threo-

nine rather than from a serine residue has been found to act as the catalytic nucleophile in some proteinases, including the putative activities of the proteosome (16), to the best of our knowledge, no functionally characterized thioesterase has been reported to use threonine in this way.

We have carried out site-directed mutagenesis targeting the putative catalytic residues and defined structures by x-ray crys-
tallography of the wild type and mutant FlK proteins both with and without bound analogues of FAcCoA. Our studies provide insights into the catalytic mechanism of FlK and the high sub-

strate specificity of FlK for FAcCoA over the closely related AcCoA.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Equipment—All oligonucleotide primers were synthesized by MWG-Biotec. Restriction enzymes were purchased from New England Biolabs. T4 DNA ligase was from Fermentas Life Sciences, and cloned Pfu DNA polymerases were from Stratagene. The following reagents were purchased from Sigma: AcCoA, 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent), CHES, ethylene glycol, polyethylene glycol 4000, polyethylene glycol monomethyl ether 2000, potassium chloride (KCl), L-selenomethionine (L-SeMet), trisodium citrate, Tris, sodium acetate, and sodium fluoride. Amino acids used for cell cultures were from DUCHefa Biochem. FAcCoA was synthesized as described previously (7). An ÄKTA FPLC system and a HiLoad Superdex S200 column (16 × 60 cm) (GE Healthcare) were used for gel filtration purification of proteins. The protein concentration was determined using the Bradford method (Sigma). DNA sequencing was performed in the Department of Biochemistry DNA Sequencing Facility (University of Cambridge) on an Applied Biosystems 3730xl DNA analyzer. Escherichia coli NovaBlue or DH10B cells were used for cloning, and E. coli RosettaTM (DE3)pLysS cells were used for protein expression.

Cloning of FlK and Mutants—The cloning of the recombi-
nant His-tagged wild-type FlK (WTFlK) in pET28a(+) (Nova-
gen) was described in our previous study (7). Mutants of FlK each containing a single amino acid mutation were created by PCR using the WTFlK-expressing plasmid as a template. For each mutant, two PCR amplifications were carried out using two primer pairs: T7-27/3'-5' primers for PCR-1 and T7T-27/ 5'-3' primers for PCR-2. The T7-27 primer (5'-TAATAGCACTCATATAGGGAATTGTG-3') and T7T-27 primer (5'-CTAGGTTATTGCTCAGCGGTGCCAGCA-3') hybridize, respectively, to the upstream or downstream regions of the multicloning site in pET28a(+). Mutations were created in either the 3'-5' primer or the 5'-3' primer or both, depending on the specific design for each individual mutant.

PCR was carried out using cloned Pfu DNA polymerase (Stratagene) with 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min plus a final extension at 72 °C for 10 min. The resulting PCR products were digested with appropriate restriction enzymes, purified by gel extraction (Qiagen), and inserted into pET28a(+) vector between the NdeI and BamHI sites. The inserts of the recombinant plasmids were verified by DNA sequencing. Constructs with the correct inserts were introduced into E. coli RosettaTM (DE3)pLysS competent cells (Novagen) for protein expression.

Expression and Purification of FlK and FlK Mutants—An overnight culture (10 ml) from a single colony harboring the desired plasmid was used for inoculation of 1 liter of fresh LB medium containing kanamycin (50 µg/ml). The culture was incubated at 37 °C, 250 rpm until A600 was 0.5–0.8 was reached. Overexpression of the proteins was induced by 0.2–0.3 mM isopropyl 1-thio-β-D-galactopyranoside overnight at 16 °C with shaking at 220 rpm before being harvested by centrifugation. For incorporation of L-SeMet into FlK, 1 liter of fresh medium containing 6 g of Na2HPO4, 3 g of KH2PO4, 1 g of NH4Cl, 1 g of NH4HCl, 0.5 g of NaCl, 1 mM MgSO4, 4 g of glucose, 0.5 mg of thiamine vitamin B1, and 50 mg of kanamycin was inoculated with cells from a 10-ml overnight culture and incubated at 37 °C with shaking at 250 rpm. When the cell density reached A600 = 0.3–0.5, a mixture of l-lysine (100 mg), l-phenylalanine (100 mg), L-threonine (100 mg), l-leucine (50 mg), l-isoleucine (50 mg), l-valine (50 mg), and l-SeMet (50 mg) was added into the culture, and incubation was continued for 30 min at 37 °C. Iso-

propyl 1-thio-β-D-galactopyranoside was then added to a final concentration of 1 mM to induce protein overexpression over-

night at 16 °C, 200 rpm. The cells were broken by sonication for 4 min (1 s on, 10 s off) to release overexpressed recombinant protein. The supernatant of the cell lysate was applied to a His-

Bind® column (Novagen) charged with Co2+ ions. Nonspecific proteins were removed by washing the column with washing buffer (0.5 M NaCl, 40 mM imidazole, 20 mM Tris-HCl, pH 7.9), and the recombinant protein was eluted with elution buffer (0.5 M NaCl, 200 mM imidazole, 20 mM Tris-HCl, pH 7.9). The buffer containing the purified proteins was exchanged with a buffer containing 100 mM KCl and 20 mM Tris-HCl, pH 7.5, and concentrated at 4 °C to a total volume of 1–2 ml. The His tag of the proteins was removed by thrombin (restriction grade; Novagen) digestion at room temperature overnight. The tag-

free proteins were further purified by gel filtration on an ÄKTA Explorer FPLC system using a HiLoadTM 16/60 SuperdexTM 200 Prep Grade column and a mobile phase containing 100 mM KCl and 20 mM Tris-HCl, pH 7.5. The purity of the proteins was checked by SDS-PAGE. The protein masses were confirmed by liquid chromatography-electrospray ionization-mass spec-
trometry on a 2.0 × 250-mm Jupiter 5-μm C4 column (Phenomenex) using a Finnigan LCQ (Thermo Finnigan) coupled with an HP1100 high pressure liquid chromatography system (Agilent) with a flow rate of 0.2 ml/min and the following mobile phase gradient: 0–5 min, 35–45% B; 5–25 min, 45–75% B; 25–30 min, 75–95% B (buffer A: 0.1% trifluoroacetic acid in H₂O; buffer B: 0.1% trifluoroacetic acid in acetonitrile).

Enzymatic Assays—The thioesterase activity of FIK and its mutants on FAcCoA was measured by monitoring spectrophotometrically the increase in absorbance at 412 nm due to the reaction of released CoASH with 5,5’-dithio-bis(2-nitrobenzoic acid with a CARY 100 Bio UV-visible spectrophotometer (Varian) as described previously (7). All assays were performed at 25 °C in a total volume of 0.5 ml in a spectrophotometer cuvette with a 1-cm light path.

Preparation of Fluoroacetyl Carba(dethia)-panthetine and Fluoroacetyl Oxa(dethia)-panthetine—To prepare fluoroacetyl carba(dethia)-panthetine (FAcCPan), monofluorination of (O,O’-diacetyl)-malonyl carba(dethia)-panthetine methylester with Selecfluor™ was carried out, followed by base-catalyzed loss of the protecting groups and concomitant deprotection and decarboxylation of the malonyl moiety. Fluoroacetyl oxa(dethia)-panthetine (FAcOPan) was obtained from 1-ethyl-3-(3-dimethylaninopropyl) carbodimide coupling of O,O’-isopropylidene-oxa(dethia)-panthetine with sodium fluoroacetate, followed by acetyl deprotection with Dowex 50-X8-400.

Crystallization of SeMet FIK in the Apo-form—The crystallization trials with the SeMet FIK protein at a concentration of 20 mg/ml in 50 mM Tris-HCl and 100 mM KCl, pH 7.5, were carried out using a Cartesian HONEYBEE™ 81 crystallization robot (Genomic Solutions), and protein crystallization was screened using the crystallization conditions from SM1 (Nextal), Classics, pHclear, and pHclear II (Qiagen) crystallization solution kits. In each reservoir of the 96-well CrystalQuick crystallization plate with square sitting drop position (Molecular Dimensions) were added 100 nl of the reservoir solution and 100 nl of protein solution at 10 mg/ml in 50 mM Tris-HCl and 100 mM KCl, pH 7.5. The best hits without acetate appeared in a condition that comprised 100 mM CHES, pH 9.5, and 1 m trisodium citrate. After optimization, the best crystals were obtained in a condition containing 100 mM CHES, pH 9.5, and 0.8 m trisodium citrate with a protein concentration of 10 mg/ml in the presence of 10 mM FAcCPan or FAcOPan.

Data Collection and Structure Determination—Data for apo-form WtFIK and SeMet FIK were collected at beamline ID14.4, European Synchrotron Radiation Facility (Grenoble, France). The data sets were processed using DENOZO and SCALEPACK (version 1.97) (17). The data were truncated and converted to structure factors using TRUNCATE (18) from the CCP4 suite (19). X-ray data for complexes of WtFIK-FAc, T42AFlK-FAc, T42SFlK-FAcCoA, WtFIK-FAcCPan, and WtFIK-FAcOPan were collected at the Swiss Light Source, and the WtFIK-FAc, T42SFlK-FAc, and T42SFlK-FAc were collected at the European Synchrotron Radiation Facility. The data sets were processed using the program Mosflm (20) and scaled by SCALA (21). The structure of SeMet FIK in its apo-form was solved using single-wavelength anomalous diffraction and the Phenix program (22), and the other structures were solved by molecular replacement using the apo-structure as probe search in the program AMoRe (23) in the CCP4 suite (19). The refinement was carried out using the program REFMAC 5.2 (24). Visual inspection and water addition were performed using XtalView/xfit (25) and Coot (26). The quality of the model was assessed using PROCHECK (27). The figures were made using PyMOL (28).

RESULTS
Site-directed Mutagenesis—To investigate the reaction mechanism of FIK, mutants of FIK were created at residues Thr42, Glu50, and His76, candidates for the catalytic triad in the FIK protein. To assess the involvement of these residues in the catalytic mechanism, the activities of the mutants were assayed and compared with that of WtFIK. Four mutants of the FIK protein carrying single amino acid mutations (T42S, T42A, E50A, and H76D, respectively) were cloned in pET28a(+) and overexpressed in E. coli Rosetta™ (DE3)pLysS cells as His-tagged proteins. All proteins were purified by Co²⁺-charged
**Crystal Structure of Fluoroacetyl-CoA Thioesterase**

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Soluble protein</th>
<th>$k_{cat}^a$</th>
<th>$K_m^b$</th>
<th>$k_{cat}/K_m^b$</th>
<th>FacCoA concentration above which substrate inhibition was observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg liter</td>
<td>s⁻¹</td>
<td>μM</td>
<td>μM⁻¹ s⁻¹</td>
<td>μM</td>
</tr>
<tr>
<td>WtFlK</td>
<td>~10–12</td>
<td>0.044 ± 0.001</td>
<td>30 ± 1.3</td>
<td>1.47 ± 0.10</td>
<td>12</td>
</tr>
<tr>
<td>T42SFK</td>
<td>~10–12</td>
<td>0.409 ± 0.159</td>
<td>15 ± 1.2</td>
<td>27.3 ± 12.8</td>
<td>19</td>
</tr>
<tr>
<td>T42AFIK</td>
<td>~2</td>
<td>0.111 ± 0.041</td>
<td>206 ± 67</td>
<td>0.54 ± 0.37</td>
<td>19</td>
</tr>
<tr>
<td>E50AFIK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The units are number of CoA released/s/enzyme molecule.

Note: The error values given for $k_{cat}/K_m$ are calculated from the sum of the relative errors on values in the numerator and denominator.

His-Bind resin (Novagen) followed by gel filtration. Both WtFlK and T42SFK were expressed as soluble proteins with a yield of over 10 mg from 1 liter of culture. T42AFIK and E50AFIK mutants yielded only 2 mg of soluble enzyme/liter of culture, although their expression levels were comparable with those of WtFlK and T42SFK. During storage at 4 °C T42SFK and T42AFIK also showed a much higher tendency to aggregate than WtFlK. The recombinant H76DFIK protein expressed in E. coli was completely insoluble, so it was not used for further studies. All of the mutations seemed to disturb protein folding, with the H76D mutant being the most affected. The molecular weights of the purified proteins were determined by liquid chromatography-electrospray ionization-mass spectrometry. Retention times on gel filtration through a Superdex S200 column indicate that purified WtFlK and the T42AFIK, T42SFK, and E50AFIK mutants are all dimers.

The enzymatic characterization of WtFlK using FAcCoA as substrate has been reported previously (7). The present study demonstrated that replacing the Thr42 with Ala42 in FIK abolishes the enzyme activity, supporting our hypothesis that the Thr42-Oγ may act as the catalytic nucleophile that attacks the carbonyl thioester carbon of FAcCoA to initiate the hydrolysis reaction. Not surprisingly, T42SFK is still able to hydrolyze FAcCoA with a $K_m$ value (15 μM, 50% lower than that for WtFlK (30 μM)). It is noteworthy that T42SFK activity was inhibited at a substrate concentration above 12 μM. These results suggest that the Ser42-Oγ in T42SFK is able to carry out the nucleophilic attack and that the absence of the Thr42-Cγ in the T42S mutant may have led to decreased ability to ensure correct substrate binding, resulting in the substrate inhibition. The $k_{cat}/K_m$ value of E50AFIK (0.54 mm⁻¹ s⁻¹) is only 37% of that for WtFlK, whereas the $K_m$ (206 μM) is much higher than that for WtFlK. E50AFIK also exhibited substrate inhibition when the FAcCoA concentration was above 19 μM, suggesting that Glu50 plays an important role in substrate recognition (Table 1).

**FIK Crystal Structure Determination**—In order to understand the catalytic mechanism of FIK, in particular how FIK distinguishes between FAcCoA and the structurally very similar AcCoA, 10 crystal structures of WtFlK, T42SFK, and T42AFIK with or without various ligands were determined. The WtFlK structures were solved in the apo-form and in complex with either the substrate analogue AcCoA or the substrate fragment analogues FAcCPan and FAcOPan, the product FAc, or the product analogue Ac. Although attempts to crystallize T42S and T42A mutants in their apo-forms failed, crystals of T42SFK in complex with Ac, FAc, or AcCoA and T42AFIK with bound FAc were obtained, and their structures were solved.

**Overall Fold and Oligomeric Association of FIK**—The crystals of FIK diffracted at resolutions between 2.35 and 1.5 Å and belong to space group C2 or P2₁ with four, or eight FIK molecules in the asymmetric unit, depending on the nature of the complex. Those complexes that crystallized in P2₁ (WtFlK-Ac, WtFlK-FAc, T42SFK-Ac, T42SFK-FAc, and T42AFIK-FAc) have a dimer in the asymmetric unit with a local 2-fold symmetry axis. The crystals in P2₁ have either dimers (two in T42SFK-AcCoA and apo-SeMet WtFlK) in the asymmetric unit, also with local 2-fold symmetry, or two tetramers (WtFlK-FAcCPan and WtFlK-FAcOPan) with orthogonal 2-fold axes giving 222 pseudosymmetry (Fig. 1, A and B). Operation of the crystallographic 2-fold axis on the dimers in the asymmetric unit in space group C2 generates a tetramer, identical to that observed in the WtFlK-FAcPan or WtFlK-FAcOPan crystal structures. The observation that these crystal structures contain discrete tetramers suggests that the protein may have dimer-tetramer equilibrium in solution in which tetramers predominate at high concentrations.

The protomer of FIK has a hot dog fold that comprises a five-strand antiparallel β-sheet (the bun) wrapping around a central α-helix (the sausage) (Fig. 1A). The hot dog fold was first observed for the structure of β-hydroxydecanoyl thiol ester dehydratase (12). A diverse range of enzymes with distinct catalytic activities has been found to have this fold. Thioesterases are important members of the hot dog superfamily and include 4-hydroxybenzoyl-CoA thioesterases from *Pseudomonas* sp. CBS-3 (29) and from *Arthrobacter* sp. SU (30), *Escherichia coli* thioesterases II (13), and acyl-CoA thioesterases YciA from *Hemophilus influenzae* (H10827) (31). Despite the conserved hot dog fold, these thioesterases share a very low degree of identity at amino acid sequence level. The FIK asymmetric unit contains a dimer with pseudoasymmetry about a 2-fold axis as observed previously in the structure of 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp. (29), and (R)-specific enoyl-CoA hydratase from *Aeromonas caviae* (32). Most other thioesterases with the hot dog fold form tetramers, although hexameric structures have also been observed (31, 33). The main contacts between the two protomers in the FIK dimer are through strand 2 (residues 70–76) of the two protomers. The β-strands of the two protomers form together a 10-strand...
FIGURE 1. Overall structure of FlK depicted in a schematic representation. A, structure of WtFlK dimer. The two protomers of the dimer are shown in green and orange, respectively. B, tetrameric structure (dimer of dimers) of WtFlK in complex with the substrate analogue FAcCPan. The FAcCPan molecule is shown as sticks. The two protomers of the dimer on the top are indicated in green and orange, respectively. A red/blue color scheme is used for the dimer at the bottom.

FIGURE 2. Catalytic center of FlK. A, the catalytic triad of FlK is composed of Thr$^{42}$, His$^{76}$ (shown as sticks), and a conserved water molecule (Wat) (shown as a sphere). Green and orange colors indicate the two protomers of the dimer, respectively. B, the catalytic triad is located at the dimer interface. The electron density map used was $2Fo-Fc$.

The tetrameric structure observed for WtFlK-FacCPan is formed by a dimer of dimers with pseudo 222 symmetry (Fig. 1B). The main interaction regions are the 10-strand $\beta$-sheets present in the dimers, which are positioned back to back. Non-polar interactions predominate, but additionally there are some water-mediated hydrogen bonds. Similar tetramers have been observed in other thioesterases (13, 30, 34, 35).

The contact area between two dimers is $\sim$3300 Å$^2$, corresponding to about 30% of the total solvent-accessible area of each dimer, further suggesting the possibility of an equilibrium between dimers and tetramer in solution.

The Active Site—Consistent with the results of the site-directed mutagenesis of FlK described above, the crystal structure of apo-form WtFlK shows that side chains of the candidate catalytic residues Thr$^{42}$ and His$^{76}$ in one protomer and that of Glu$^{50}$ from the adjacent protomer cluster between the dimer interface into the region between the “bun” and the “sausage” (Fig. 2, A and B). As expected, the O$_{\alpha}$ of Thr$^{42}$ and the N$_{\epsilon1}$ of His$^{76}$ are within hydrogen bonding distance (2.74 Å). To our surprise, we observed that the carboxyl group of Glu$^{50}$ is located on the opposite side of Thr$^{42}$, where it cannot have any direct contact with His$^{76}$. However, a water molecule (Wat$^1$) is in a perfect position to form a hydrogen bond with the N$_{\epsilon2}$ of His$^{76}$ (2.79 Å). This water molecule is conserved in the crystal structures of wild-type and mutant FlK with or without bound ligands. Although this observation was unexpected, it is in good agreement with our site-directed mutagenesis results. A water molecule replacing the acidic residue in this type of catalytic triad was also found in the HAV-3C gene product, an $\alpha$-chymotrypsin-like protease produced by the hepatitis A picornavirus (36). The Thr$^{42}$-His$^{76}$-Wat$^1$ network is further stabilized by a hydrogen bond between Wat$^1$ and Thr$^{80}$ side chain hydroxyl and another conserved water molecule (Wat$^2$) that also forms a hydrogen bond with the Phe$^{40}$-N. Around the His$^{76}$-Wat$^1$-Thr$^{80}$/Wat$^2$ network, the side chains from Val$^{39}$, Phe$^{30}$, Ala$^{78}$, Ala$^{79}$, and Ile$^{113}$ form a hydrophobic shield, protecting the network protomer, suggesting strong interactions between them. These observations have led us to assume that the minimal active unit of FlK is a dimer.
from attack by solvent molecules. Based on our structural evidence, we now propose that the catalytic triad in FlK comprises Thr42-His76-water (Fig. 2A), located at the interface between two protomers with two active sites in each FlK dimer (Fig. 2B).

Substrate Binding Pocket—Although attempts to co-crystalize FlK with FAc and CoA failed, we successfully obtained crystals of T42SFlK with bound AcCoA and WTFlK in complex with two analogues of fluoroacetyl-pantetheine, FAcCPan, and FAcOPan. The sulfur atom of the thioester is replaced by a methylene in FAcCPan and by an oxygen atom in FAcOPan (Fig. 3). Comparison of the electron density maps of the crystals obtained from co-crystallization of WTFlK with FAc and CoA indicates that only FAc was bound. Comparisons of the structure of WTFlK without bound ligand with those of WTFlK-FacCPan, WTFlK-FacOPan, and T42SFlK-AcCoA have provided interesting insights into the structural basis of substrate recognition by FlK.

In the ligand-free WTFlK crystal structure, three well conserved water molecules (designated Wat3, Wat4, and Wat5) mediate a hydrogen bonding network linking Thr42 and Gly43 in one protomer to Glu50, Arg120, and Gly69 in the neighboring protomer; Wat3 links the Gly43-N with the Glu50-O/H9280, and Wat4 and Wat5 are within hydrogen bonding distance of each other and of the Thr42-N and Gly69-N, respectively. Converting Arg120 to Ala120 yielded completely insoluble protein, as did converting Glu50 to Ala50, suggesting the importance of these interactions in maintaining the correct protein folding and/or the correct architecture of the active site cavity.

In the T42SFlK-AcCoA complex, the AcCoA is sandwiched between the long -$\beta$-sheet and residues 17–42 that form two small -$\alpha$-helicities (Fig. 4B). However, only the acetyl and $\beta$-mercaptoethylamine moieties from acetyl-CoA are buried in the protein, whereas the pantetheinic acid and the 3'-phosphoryl-ADP are exposed to solvent, allowing considerable flexibility (Fig. 4, A and B). Three of the four protomers present in the asymmetric unit have a bound AcCoA, and in one of them, it was possible to fit the complete molecule, despite the poor electron density for the 3'-phosphoryl-ADP moiety, possibly due to the high flexibility in the solvent. The overall structure of FlK does not change significantly as a result of the T42S point mutation and the binding of AcCoA, but the AcCoA has forced Wat4 and Wat5 out of the active site cavity. The acetyl methyl group of the AcCoA occupies the position of Wat5, and the thioester carbonyl oxygen interacts via a hydrogen bond to Ser42-N. The orientations of the three bound AcCoA molecules at the active site are significantly different, reflecting a dynamic AcCoA-FlK interaction process and the flexibility of the substrate binding pocket.
and/or Gly\textsuperscript{69}-N in a seemingly random manner in terms of hydrogen bond donor-acceptor matching, suggesting that the backbone amide groups of Thr\textsuperscript{42} and Gly\textsuperscript{69} may be crucial binding interactions available at the active site to bind the substrate.

**Computational Docking of FAcCoA to FIK**—We have modeled a FAcCoA molecule into WtFIK (Fig. 5), based on the crystal structures of FIK in complex with various ligands, especially with AcCoA. The minimum energy was calculated using the SYBYL 8.1.1.09097 program. In a similar way to the AcCoA bound in T42SFIK, the 3'-phosphoryl-ADP region of the docked FAcCoA is at the enzyme surface in front of the entrance of the tunnel leading to the active site. The rest of the molecule extends into the tunnel, where the pantetheinyl moiety interacts through its N\textsubscript{12}, O\textsubscript{9}, N\textsubscript{14}, and C\textsubscript{19}, respectively, with the main chain oxygen and the side chain of His\textsuperscript{76}, Ala\textsuperscript{79}-N, and Phe\textsuperscript{128}. The hydrogen bond between the side chains of Arg\textsuperscript{120} and Glu\textsuperscript{30} is broken due to the flip of the carboxyl group of the Glu\textsuperscript{50}. The fluorine is placed at a hydrogen bonding distance from Gly\textsuperscript{69}-N and the guanidium group of Arg\textsuperscript{120}. Mutation of the Arg\textsuperscript{120} to Ala\textsuperscript{120} yielded a completely insoluble protein (data not shown), consistent with the involvement of Arg\textsuperscript{120} in maintaining the conformation of FIK. Thr\textsuperscript{72}-O\textsubscript{y} is now located closer (3.79 Å) to the thioester carbonyl carbon and at a better, although not optimal, position for a possible nucleophilic attack. The Thr\textsuperscript{42}-N that is close (2.81 Å) to the thioester carbonyl oxygen could act as an oxyanion hole for stabilization of the tetrahedral acyl-enzyme intermediate (Fig. 5).

**Increased Flexibility at the Active Site Resulting from Mutations of Thr\textsuperscript{42}**—The hydrophobic interactions between Thr\textsuperscript{42}-C\textsubscript{y2} and Ile\textsuperscript{72}-C\textsubscript{y2} in the adjacent protomer provide a “dry” environment around the catalytic O\textsubscript{y1} of Thr\textsuperscript{42}, protecting it from attacks by solvent molecules, a feature observed for other enzymes that have similar catalytic triad mechanisms (37). The Thr\textsuperscript{42}-C\textsubscript{y2} may also restrain the freedom of the substrate in the active site. Loss of this restraint imposed by the C\textsubscript{y2} in the T42S mutant may allow random misbinding of the substrate, which in turn may result in the substrate inhibition observed in the enzyme assays. In support of this notion, the imidazole group of the His\textsuperscript{76} in the complex T42AFIK-FAc has a rotational disorder of ~90° or exists in two alternative conformers not observed in WtFIK structures, and the Ser\textsuperscript{42} in one of the protomers of the T42SF1K-FAc structure shows a double conformation, demonstrating the increased flexibility of these residues in the absence of the Thr\textsuperscript{42}-C\textsubscript{y2} and/or -O\textsubscript{y2} (Fig. 6).

**Conformational Change for Product Release**—The positions of the main chains are almost completely conserved in all FIK structures with or without mutations and/or bound ligands. Local conformational changes, especially in the hydrophobic loops \textsuperscript{33}FAEF\textsuperscript{37}, are observed. The side chain conformations of Phe\textsuperscript{33}, Phe\textsuperscript{36}, and Pro\textsuperscript{37} in the structures with bound Ac or FAc...
DIFFUSION

FlK, a thioesterase from fluoroacetate producing S. case
tyly, hydrolyzes the thioester bond of FAcCoA but not that
of AcCoA. The selectivity of FlK to distinguish FAcCoA from
AcCoA is remarkable because it ensures that AcCoA, a key
intermediate of the primary metabolism is not cleaved by
FlK. The selectivity of FlK to distinguish FAcCoA from
that of either type I or type II thioesterases. FlK has a hot dog fold but uses an active site completely different
from that of either type I or type II thioesterases. FcoT has
a tetrahedral transition intermediate. The positive charge
attacked by the nucleophile as the Thr42-O\(^{-}\) in WtFlK. When FAcCoA concentration was higher than 12 \(\mu\)M, however, excess-substrate inhibition (38) was observed for the mutant. In other words, high concentrations of FAcCoA inhibited the activity of
T42SFlik. We speculate that the absence of the Thr42-C=O in
T42SFlik may have created more space, allowing an easier
access of substrate to the active site, especially at low sub-
strate concentrations, giving rise to the lower \(K_m\) value and
the higher turnover of T42SFlik reaction as compared with
WtFlK activity. However, the same mutation also increased
protein flexibility, as shown by the double confirmations
of the active site residues in some of the protomers of the
mutant (Fig. 6). The enzyme-substrate complex formed be-
tween these abnormal active sites and substrate could be non-
productive. At high substrate concentrations, the chance
of non-productive enzyme-substrate complex formation increases when more and more normal active sites are occupied
by FAcCoA. The formation of non-productive enzyme-sub-
strate complex at the active site of one protomer may also inter-
fere with the catalytic activity of the partner protomer of the
same dimer. These may explain the excess-substrate inhibition
phenomenon observed for T42SFlik and E50AFlik, but the exact
mechanism remains to be elucidated.

FlK is a homodimer with a hot dog fold similar to other
type II thioesterases, although it does not show extensive
sequence similarity to any type II thioesterases with known
function. A common feature shared by nearly all functionally
characterized hot dog fold thioesterases is the presence of an
acidic residue, aspartate or glutamate, providing general base catalysis. One exception is FcoT, a long-chain fatty acyl-
CoA thioesterase from Mycobacterium tuberculosis, which has a hot dog fold but uses an active site completely different
from that of either type I or type II thioesterases. FcoT has
been proposed to represent a new class of thioesterases, type
III thioesterases (39). With a type II thioesterase fold, the
catalytic machinery of FlK (Thr-His-water) resembles those of
type I thioesterases (Ser/Cys-base-acid), exhibiting an
unconventional combination of protein fold and catalytic
mechanism. Superposition of FlK with 18 structures of type
II thioesterases from the Protein Data Bank reveals that the
Glu\(^{50}\) in FlK is well conserved in most of these thioesterases,
although in some cases, it is an aspartate instead of a glutamate (data not shown).

Among all of the hot dog fold proteins in the Protein Data Bank, only two hypothetical proteins, TTHA0967 (Protein Data Bank entry 2CWZ) from Thermus thermophilus and TM0581 (Protein Data Bank entry 2Q78) from Thermotoga maritima, have shown head-to-tail sequence similarity to FIK (33 and 22% identity, respectively). Superposition of FIK with these two structures has revealed a very similar putative active site in 2CWZ and 2Q78. Most of the residues lining the active site of FIK, Thr^{42}, Glu^{50}, Gly^{69}, and His^{76}, are well conserved in 2CWZ (Thr^{36}, Glu^{44}, Gly^{48}, and His^{70}) and in 2Q78 (Thr^{38}, His^{55}, and Arg^{57}) with the following differences. First, the counterpart of the Arg^{120} of FIK in 2CWZ is a glutamine (Gln^{115}). The Arg^{120} in the unliganded FIK interacts through its guanidinium group with the carboxyl side chain of Glu^{50}. The amide side chain of Gln^{115} in 2CWZ, however, is pointing in the opposite direction with no possibility of forming any direct contact with Glu^{44}. A large empty space can be observed in the putative active site of 2CWZ due to this change of Arg to Gln. If 2CWZ is also an acyl-CoA thioesterase, the substrate should have a larger acyl moiety than a fluoroacetyl group. Second, the equivalents of FIK Glu^{50} and Gly^{69} in 2Q78 are His^{46} and Val^{65}, respectively. The imidazole side chain of the His^{46} and the iso-propyl group of the Val^{65} at the active site provide a binding motif quite different from that in FIK.

The significant differences in the overall positions of FAccPan and FACOPan in the active site of FIK as compared with that of the acetyl-pantetheinyl segment of the T42SFK-bound AcCoA (Fig. 3) suggest that binding of the nucleotide portion of the substrate on the enzyme surface dictates the depth to which the fluoroacetyl pantetheinyl arm extends into the active site cleft, ensuring the correct interaction between the arm with the amino acid residues in the vicinity of the active site. Although the sulfur atom of the thioester is considered a poor hydrogen bond acceptor, the transient dipole interaction between the sulfur and the main chain amides along the substrate binding tunnel may also contribute to positioning of the substrate. In the absence of the bulky nucleotide tail and the sulfur atom, FAccPan moved further into the active site. FACOPan is retained near the tunnel entrance because the oxygen atom replacing the sulfur atom has a stronger tendency to interact with the main chain amides of the substrate binding tunnel.

Although the 3′-phosphoryl ADP tail of FAccCoA is required for the overall positioning of FAccCoA in the FIK active site, the accurate orientation of the fluoroacetyl thioester moiety must be fine tuned by the hydrogen bonding of the fluorine with the side chain of Arg^{120} and the main chain amide of Gly^{69} in order to assume the required catalytic coordination. The guanidinium group of Arg^{120} and the main chain amide of Gly^{69} appear to be the key determinants for the unusual high substrate specificity of FIK, which serve to recognize the only structural difference between FAccCoA and AcCoA through their interactions with the fluorine of FAccCoA. Moreover, the thioester carbonyl carbon in FAccCoA is more positively charged than that in AcCoA due to the strong electronegativity of fluorine. The interactions between the fluorine and Arg^{120}/Gly^{69} also help to stabilize this positive charge, making the carbonyl carbon a better target for nucleophilic attack by Thr^{82}·O\(_{\text{γ}}\) (Fig. 5).

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REFERENCES


Crystal Structure of Fluoroacetyl-CoA Thioesterase
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Structural Basis for the Activity and Substrate Specificity of Fluoroacetyl-CoA Thioesterase FIK

Marcio V. B. Dias, Fanglu Huang, Dimitri Y. Chirgadze, Manuela Tosin, Dieter Spiteller, Emily F. V. Dry, Peter F. Leadlay, Jonathan B. Spencer and Tom L. Blundell


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