Of barn owls and bankers: a lush variety of α/β hydrolases

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α/β Hydrolase fold proteins are an important, diverse, widespread group of enzymes not yet fully exploited by structural biologists. We describe the current state of knowledge of this family, and suggest a smaller definition of the required core and some possible future avenues of exploration.

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Are a barn owl and a Wall Street banker related by divergent evolution just because both are rapacious? Was swooping down on unsuspecting victims to make a killing intrinsic to their common ancestor? Did it arise independently? Or is there some other feature that shows they are related — like the five digits in their claws? This is precisely the conundrum posed by the α/β hydrolase fold family, a family where all the members are related by divergent evolution and yet where — unlike the serine proteases — the level of divergence is extremely high and the structural similarity between related proteins is often very low.

In the early 1990s, five apparently unrelated hydrolases turned out to have the same protein fold, which we named the α/β hydolase fold [1]. The only features in common were a few aspects of structure and reaction: the sequences were unrelated; they did not operate on similar substrates; nor did they use the same nucleophile (Figure 1). In the 48 α/β hydrolase fold structures solved since then, this pattern has repeated itself: the α/β hydrolase fold is arguably the most plastic of protein folds, tolerating large insertions into a single-domain protein so that the domain size can vary from as little as 197 residues in *Fusarium solani* cutinase (FsCUT) to 582 residues in mouse acetylcholinesterase (mACE). (This is rather different than the α/β barrel enzymes, where the eight-stranded barrel is usually preserved with the addition of other complete domains.) Understanding this plasticity is important both for protein design and for protein classification efforts based on sequence.

The features of an α/β hydrolase protein

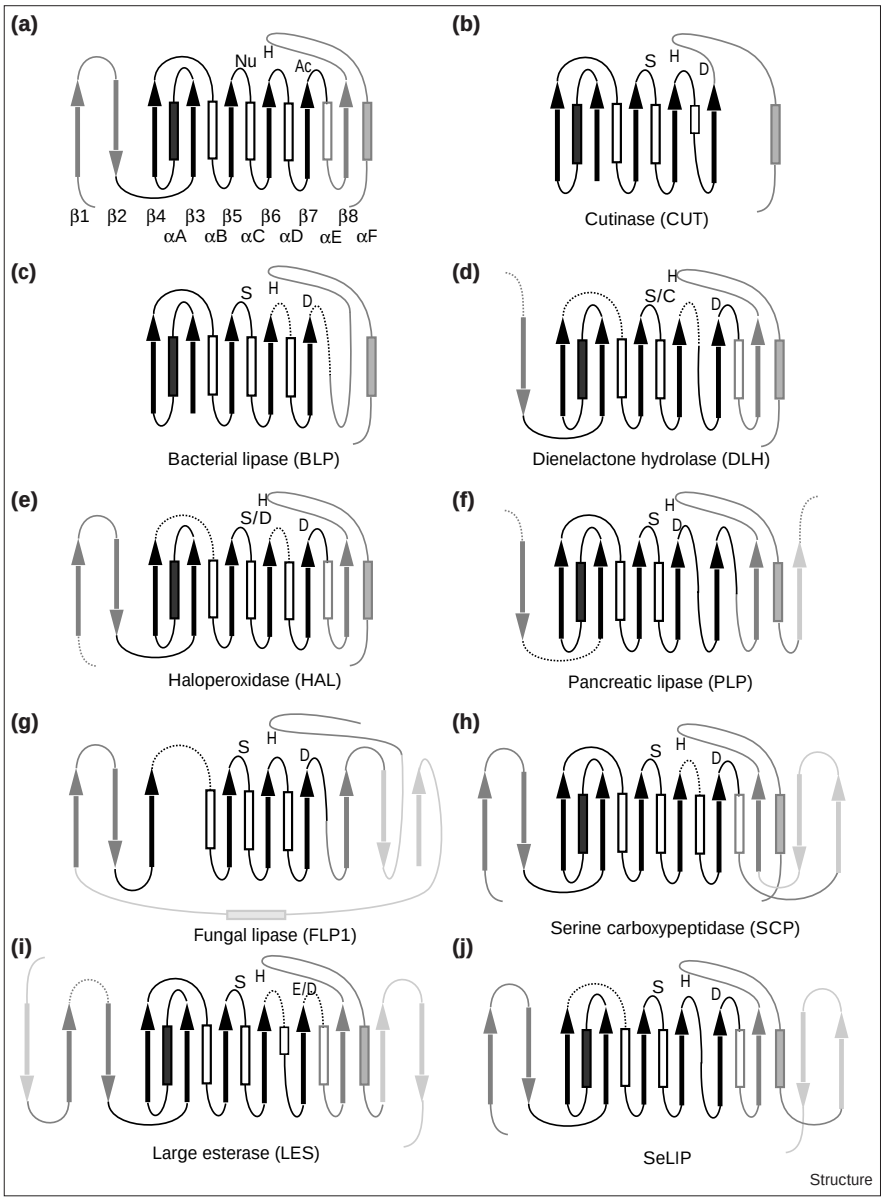
The canonical structure

We described the canonical α/β hydrolase fold as an eight-stranded mostly parallel α/β structure [1]. This description has been adopted by several databases: SCOP (http://scop.mrc-lmb.cam.ac.uk/scop/) [2]; CATH (http://www.biochem.ucl.ac.uk/bsm/cath/) [3]; and the α/β hydrolase fold database ESTHER (http://www.ensam.inra.fr/cholinesterase/) [4]. The canonical fold has strand β2 antiparallel to the rest, with the connection of the strands being +1, +2, –1x, +2x, +1x, +1x (strand order 12435678; Figure 1), and a substrate-binding crevice at the cross-over connection on strand β5. The sheet is highly twisted and bent so that it forms a half-barrel with the first and last strands approximately at 90° to one another (Figure 2a). In the canonical protein, of which *Pseudomonas fluorescens* carboxyl esterase (PfCES) [5] is a good example, the strands are connected by α helices A–F. The first and last helices (αA and αF) pack onto the convex face of the half-barrel, whereas helices αB–αE pack onto the concave surface (Figure 2a). The structurally conserved helices occur immediately before the conserved β strand; the structural module is α–turn–β, not β–turn–α. Insertions occur after one or more of the β strands β3, β4, β6 and β7. Insertions following β8 are also common, but here the insertion can occur either before or after αF. The substrate-binding domains are often made of several insertions and form a cap on top of the active site, but they can also be large individual domains, as in the C-terminal domain of pancreatic lipases (PLPs). Finally, many, but not all, of the α/β hydrolase fold enzymes have a left-handed cross-over following strand β8.

Enzymatic properties

The catalytic triad — nucleophile, acid and a histidine — form the basis of the enzymatic properties of the α/β hydrolases. Unlike the other protease families, but like the NTN (N-terminal nucleophile) hydrolase fold [6], the nucleophile is not the same in each enzyme: it can be serine, cysteine or aspartate (e.g. in haloalkane dehalogenase, HAL). The nucleophile is located in a tight turn after β5 called the 'nucleophile elbow', which is the best conserved feature and can be used as a peg for superimposing the α/β hydrolase structures (Figure 2b). The α/β hydrolase fold family includes the only examples of enzymes in which glutamate is utilised as the acid in the triad. The twist of the central β sheet allows short pieces of peptides to bring the triad residues together to form a catalytically active enzyme and creates good geometry for the 'oxyanion hole'; the oxyanion residues are usually located on strands β5 and β3.

Figure 1



Topological diagrams of the canonical, minimal and variations of the α/β hydrolase fold. The minimal fold is indicated in black, the canonical fold in dark grey, and extensions to the core beyond the canonical fold are shown in light grey. Strands are shown by arrows and helices by rectangles. Filled rectangles are below the plane of the β sheet (on the convex surface); open rectangles are above (on the concave surface). (a) The canonical fold [1] and minimal fold, with the strands and helices labelled and the catalytic triad marked: Nu, nucleophile; Ac, acid; H, histidine. The schematics for each α/β hydrolase fold family show the catalytic triad in single-letter amino acid code and possible (but not required) excursions with a dotted line. (b) The CUT family. The structure of one family member, *Fusarium solani* cutinase (FsCUT) [9], has been determined. This is the smallest α/β hydrolase fold enzyme. (c) The BLP family comprises bacterial lipases, such as *Pseudomonas cepacia* (PcBLP) [14], and the rubber tree hydroxynitrile lyase (rtHNL) [15]. These are somewhat large enzymes with an irregular structure instead of αE and β8 and a unique insertion between β7 and the acid residue, except in rtHNL. (d) The DLH family includes porcine proline oligopeptidase (pPOP) [16], *Pseudomonas* sp. B13 dienelactone hydrolase (P13DLH) [17] and *Pseudomonas fluorescens* carboxylesterase (PfCES) [5]. *Candida antarctica* lipase (CaFLP3) [18] is also a member of this family although it lacks αE instead of αD. (e) The HAL family includes a wide variety of different enzymes, such as haloalkane dehalogenases (e.g. *Xanthobacter autotrophicus* HAL [19]), epoxide hydrolases from *Agrobacterium radiobacter* (ArEHY) and mouse (mEHY), bacterial bromo- and chloroperoxidases, proline iminopeptidase from *Xanthomonas campestris*, and thioesterase from *Vibrio harveyi*. Members of this family demonstrate poor conservation at the nucleophile elbow. *Bacillus subtilis* Brefeldin A esterase (BsBES) [8] is also included in this family, although it lacks αD. (f) The PLP family includes human [20] and other mammalian PLPs. PLPs have the acid residue on β6 and a large C-terminal domain. (g) The FLP1 family includes lipases from *Penicillium caembertii*, *Humicola lanuginosa* and *Rhizomucor* sp. (e.g. *Rhizopus niveus*, RnFLP1 [21]). Strand β3 and helices αA and αE appear to be missing and an antiparallel strand precedes the catalytic histidine. The N-terminal insertion provides a helix that structurally replaces αF. (h) The SCP family includes serine carboxypeptidases from, for example, wheat (wSCP) [22], the yeast kex1 protein and human protective protein. Two additional strands are present between αE and β8. (i) The LES family includes acetylcholinesterases (e.g. *Torpedo californica* ACE, TcACE [23]), fungal lipases (e.g. *Geotrichum candidum* FLP2, GcFLP2 [24]) and the bovine bile-salt-activated cholesterol esterase (bBACE) [25]. This family includes the largest α/β hydrolases and large insertions follow β1, β6 and β7. Except in bBACE, the triad acid is glutamate. (j) *Streptomyces exfoliatus* lipase (SeLIP) [26] has a unique C-terminal extension and is classified separately.

Finally, the stereochemistry of attack is the mirror image of that seen in the serine protease and subtilisin families [1].

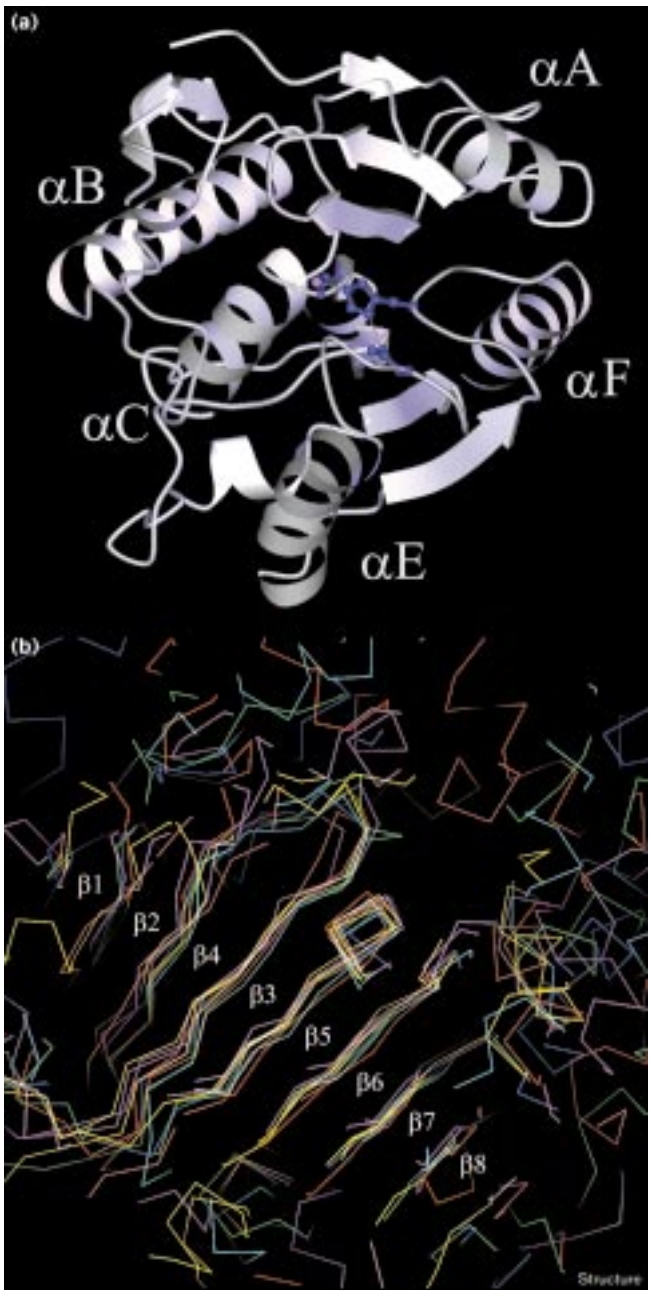
Relatively speaking

The α/β hydrolase superfamily can be arranged into families that are clearly related by sequence identity over the whole molecule (siblings), groups of families with only partial (and lower) sequence identity (cousins), and extended groupings where the sequences only match around the nucleophile elbow, if at all (distant relatives).

Siblings

The family of large esterases are siblings, with ~30% sequence identity across most or all of the molecule.

Figure 2



The typical α/β hydrolase fold. (a) The catalytic triad of α/β hydrolases viewed from the top of the mostly-parallel β sheet in *Pseudomonas fluorescens* carboxyl esterase (PfCES) and lit from the nucleophile. (b) A superposition of the common core of representative α/β hydrolase fold enzymes from Figure 1. The structures superimposed in the figure are FsCUT, PcBLP, P13DLH, XaHAL, hPLP, RnFLP1, wSCP, TcACE and SeLIP.

Even here precise distinctions must be made; we group the fungal type B lipases (FLP2s; SCOP family 3.56.9.2) with the acetylcholinesterases (ACEs; SCOP family 3.56.1) because of the high sequence identity (e.g. 30% between *Torpedo californica* ACE and *Geotrichum candidum* FLP2), because they have very similar structures (Figure 1), and because almost all have the same unusual Ser-His-Glu catalytic triad. Unfortunately, SCOP [2] groups all the fungal lipases together separate from ACE, presumably by catalytic function and origin. Despite the high sequence identity ACEs and FLP2s have significant differences in substrate preference and binding affinity: ACE has been optimised for the diffusion-controlled catalysis of a small substrate, whereas the same structures in FLP2 form the lid that achieves interfacial activation, so that the lipases are only active in the presence of macroscopic lipid surfaces.

Cousins

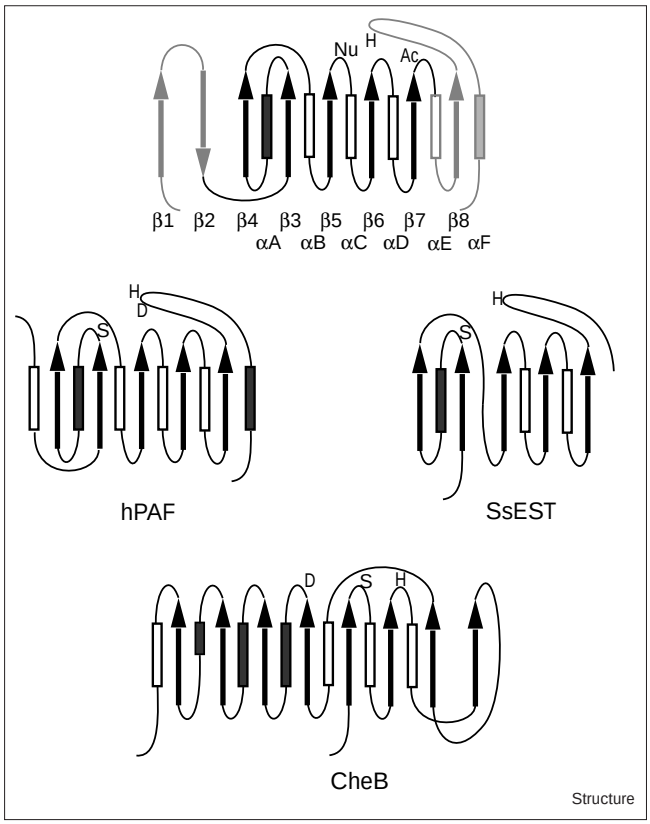
The similarity between epoxide hydrolases (EHYs) and HAL provides a striking example of cousins. EHYs are involved in the detoxification of harmful aromatic compounds in mammals. The structures of two EHYs have recently been solved — from *Agrobacterium radiobacter* (ArEHY) [7] and mouse (mEHY) (DW Christianson, personal communication). The sequence identity between ArEHY and *Xanthobacter autotrophicus* HAL (XaHAL) is 24%, making it hard to construct an accurate model of either cousin from sequence alignment alone. As in HAL, there is an α-helical insertion between strands β6 and β7 in EHYs, which partially caps the active-site cavity and contributes to the hydrophobic binding pocket. The catalytic residues are $Asp333^{nuc}$-His523-Asp495 (mEHY) and $Asp107^{nuc}$-His275-Asp246 (ArEHY), where the last residue of the triad is the least important. Intriguingly, the N-terminal domain of mEHY (not present in ArEHY) has a six-stranded all-parallel α/β structure that is a vestigial haloacid dehalogenase. Two different domains, both involved in haloalkane degradation, were thus fused into a single gene to form mEHY, with the haloacid dehalogenase becoming non-functional and vestigial. In mEHY, this domain forms a domain-swapped dimer (DW Christianson, personal communication).

Also instructive is the relationship between pig proline oligopeptidase (pPOP), which degrades short peptides, and *Pseudomonas* dienelactone hydrolase (P13DLH). The C-terminal domain of pPOP, like mEHY, is a canonical α/β hydrolase fold with structural similarity to P13DLH; the sequence identity from the structural alignment is 22%. The N-terminal domain of pPOP is an unusual seven-bladed β-propeller domain, the function of which is to prevent all except small peptides reaching the active site. P13DLH, on the other hand, uses two short peptides to bind its relatively small substrate.

Distant relatives

The structure of *Bacillus subtilis* Brefeldin esterase (BsBES) [8], which hydrolyses a macrocyclic fungal toxin, is related by sequence to the important human hormone-sensitive lipase (hHSL), which performs the rate-limiting

Figure 3



Topology diagrams of α/β hydrolase fold 'in-laws'. The canonical and minimal fold of the α/β hydrolase fold are shown at the top for comparison. Shading of helices is as described in Figure 1. Shown are human brain platelet-activating factor acetylhydrolase, isoform 1b (hPAF) [14], *Streptomyces scabies* esterase (SsEST) [13], and chemotaxis regulator protein CheB from *Salmonella typhimurium* [27].

step of hydrolysing fat stored in adipocytes. The fold of BsBES (and by inference the unsolved structure of hHSL) is closest to that of the HAL family. However, BsBES has an unusual α-helical N-terminal extension as well as a more standard insertion after β6. The latter distorts αD in BsBES. The catalytic triad of BsBES is Ser202-His338-Asp308, and allowed the authors to identify the catalytic triad of hHSL as Ser423-His733-Asp703. Despite the very low sequence homology, a fairly accurate model of hHSL had been built earlier from a more distant relative, *Candida rugosa* FLP2.

Classifying FsCUT [9] is harder still. The function of this enzyme is closely related to that of lipases and so should have evolved from the large esterase (LES) family. However, FsCUT lacks β1, β2, β8 and αE of the canonical fold. In addition, the catalytic histidine is now located in a loop-like structure that replaces the omitted αE and β8 and that is followed by a bent and distorted αF. FsCUT does, however, have some α/β hydrolase fold features: the nucleophile elbow on canonical strand β5, the inverted geometry of attack as compared to the serine proteases, and the correct core topology. The SCOP database places FsCUT with flavodoxin (α/β class 3.14.7), which also has a three-layered α/β/α fold with strand order 21345. However, FsCUT is clearly an α/β hydrolase fold protein, as there is no mechanistic relationship between cutinase and flavodoxin.

A minimal requirement

Given the wide variety of structures (Figures 1 and 2), can one construct an identikit to distinguish α/β hydrolase fold proteins from others? There seem to be four requirements. Firstly, the sequence order of the catalytic triad is nucleophile-acid-histidine, with the nucleophile on canonical strand β5. Secondly, there is a nucleophile elbow at the top of canonical strand β5, with a sequence pattern that is often Gly-X-Nuc-X-Gly (Figure 2). Thirdly, the structure starts at strand β3 and is at least five strands long including the cross-over connection at the nucleophile (strands 43567; Figure 1b). Fourthly, a long loop at the end of strand β7 allows the sidechains of the triad residues to hydrogen bond. Of the last three features, at least two are present in all α/β hydrolase fold enzymes.

Alarums and excursions

The diverse structures presented above suggest that a distinguishing feature of the α/β hydrolase fold could be its ability to be modified while retaining catalytic function. Attached to this hydrolytic domain are different substrate-binding loops with sizes that vary from simple turns to 80-residue insertions. In general, these loops are integrated into the single-domain structure, rather than added on as complete domains. The skeleton of the α/β hydrolase fold also tolerates radical changes. For instance, the serine carboxypeptidases (SCPs) have an antiparallel hairpin loop between strands β7 and β10: strand β10 in wheat SCP (wSCP) corresponds to strand β8 in the canonical fold. Apparently, during the course of evolution, two additional strands have been inserted into the domain without losing activity. Intriguingly, the topology of *Streptomyces exfoliatus* lipase (SeLIP) is similar to that of wSCP, but the sequence position of the inserted hairpin loop is different (Figure 1).

Similarly, among the five classes of α/β hydrolases that include lipases (Figure 1), many different kinds of changes are seen: the fold of bacterial lipases starts at strand β3; *Candida antarctica* lipase (CaFLP3; also misgrouped by SCOP with fungal lipases) starts at strand β2; LES family lipases have the complete canonical fold; and the PLPs contain eight β strands with an additional strand before the separate C-terminal domain. This is consistent with the idea that the minimal fold (and possibly the evolutionary ancestor) started from strand β3, but not with the idea that the α/β hydrolase fold is a two-domain protein [10]. In PLPs,

the acid of the catalytic triad is located on canonical strand β6 (Figure 1). The relationship of the FLP1 family to the other lipases is more problematic. In FLP1s (Figure 1) [11], the strand cross-over point has disappeared, suggesting that strand β3 rather than strand β4 has been lost. The catalytic triad acid residue then appears as expected after canonical strand β7, but (as in the SCP family) there is a large insertion at the end of the sheet, in this case an antiparallel strand preceding the catalytic histidine.

α/β Hydrolase fold in-laws

There are enzymes that, continuing the family analogy, are α/β hydrolase fold 'in-laws': they may, in time, be accepted into the family but should, at present, be regarded with suspicion. Chief among them are esterases such as *Streptomyces scabies* esterase (SsEST) [12] and human brain platelet-activating factor acetylhydrolase 1b (hPAF) [13] (Figure 3). The topology of these enzymes is similar to that of FsCUT, and SCOP classifies them, like FsCUT, as five-stranded flavodoxin-like molecules. The only feature in common with the α/β hydrolase fold enzymes is the sequence order of the catalytic residues (nucleophile-acid-histidine). In other respects, they are different: the nucleophile is on the equivalent of canonical strand β3, not strand β5 (Figure 3); the structure of the nucleophile elbow is different; and both acid and histidine follow the equivalent of strand β7. Furthermore, the oligomeric behaviour of hPAF suggests that it should be regarded as a G protein manqué, as it forms G protein like heterotrimers [13]. Other proteins, such as the chemotaxis protein CheB (Figure 3), are clearly not related despite the presence of the catalytic triad, because neither the topology nor the order of catalytic residues is preserved.

Barn owls revisited

Clearly, the α/β hydrolase fold superfamily is one of the more plastic protein folds discovered so far. Unlike most superfamilies, where the size of the fold varies over a rather narrow range, the fold of this family ranges widely (from 197 to 583 residues). This presents both opportunities and challenges for the structural biologist. The fold is a natural (but under-exploited) choice for investigating protein evolution, loop insertion mechanisms, protein thermostability and protein folding. For instance, what distinguishes α/β hydrolase fold enzymes from others? Are FLP1s and FsCUT α/β hydrolases? If so, why not hPAF, which has exactly the same strand topology as FsCUT? In terms of our initial analogy: is topology like rapaciousness, a superficial characteristic, or is it of deeper significance, like a pentadigital limb?

This raises another question. Are there molecular fossils to help explain how these proteins evolved? Were the large insertions borrowed from other protein(s), or did they emerge *sui generis*? Our preliminary results suggest that the source of the loops can sometimes be located.

Perhaps the fundamental question has yet to be addressed. Are the processes at work in the α/β hydrolase fold unusual? In the large part we know that α/β hydrolase fold enzymes are related because of the conserved catalytic framework. In the absence of this framework, the preserved feature might be the topology of FsCUT. Is this topology sufficient to classify two proteins as being related? The example of hPAF suggests not. This is the protein version of the rock concert problem: although you walk alone, someone there is quite likely to be related to you. The question is who — and how would you identify them by appearance alone? And — of course — they're probably bankers.

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