IIF Periodic Report: Publishable Summary Final Report

Project number:255500

TIMCat - Novel catalytic function in a TIM-barrel scaffold

The aim of this project is to examine the role of loops in enzyme evolution and function.

Concerns about the comparatively low stability of the originally proposed target enzyme, monoTcTIM, thought to be due to the exposure of hydrophobic surface on monomerisation of the native TcTIM dimer, led to a comprehensive bioinformatic examination of structurally characterised TIM proteins to find potential alternative scaffolds. The search targeted monomeric proteins with an un-elaborated TIM-barrel structure that does not have significant extensions interacting with the C-terminal face of the barrel. From over 1000 TIM-barrel structures in the PDB, only three protein scaffolds were identified which met the defined criteria – these were indoleglycerol phosphate synthase, 4-hydroxy-2-oxoglutarate aldolase and phosphoribosylanthranilate isomerase (PRAI). Of these, PRAI looked to be the best candidate. The gene for PRAI from *E. coli* was cloned, over-expressed and the protein purified. Unfortunately, the activity assay for this enzyme was found to be unsuitable for screening in microdroplets due to the small signal change on successful reaction, which was further complicated by the intrinsic chemical instability of the enzyme substrate. Consequently, the research focus shifted to examining the role of loops in enzyme function of alternative scaffolds, and in particular on developing new methodologies for manipulating loops in proteins. Concurrently, work on microdroplets has focused on developing their usability and utility for protein engineering studies.

Transposon workflow overview

New methods have been developed for a) variable-length deletion scanning and b) protein split site scanning (see Fig. 1). Both of these methods have a unified first stage, in which transposition is exploited to produce a library of a gene of interest in which each carries a randomly located blunt double-stranded break that facilitates subsequent molecular biology steps. Transposition is an important mechanism for natural evolution, but also serves as a convenient molecular biology tool due to its ability to insert a DNA cassette randomly and with low sequence preference throughout a piece of target DNA.

a) Variable-length deletion scanning

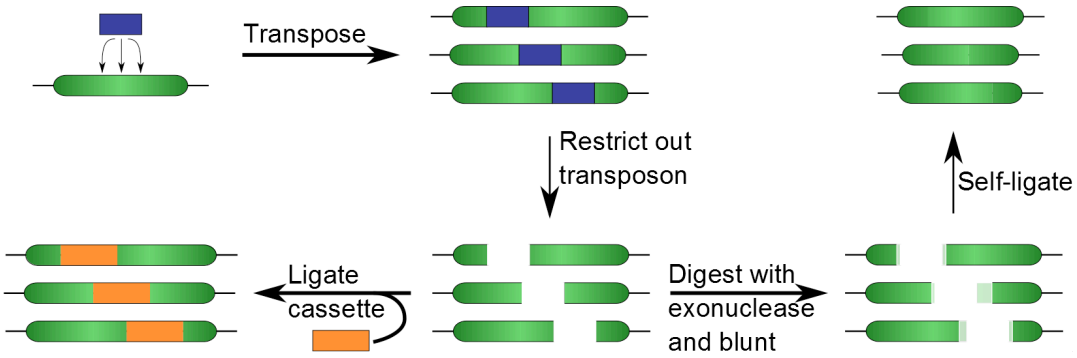

Figure 1. Transposition-based workflows. Random insertion of the transposon TransDel (blue) into a gene of interest (green), followed by excision of the transposon by restriction digestion affords a library of randomly cut copies of the gene of interest. This library can then be directly ligated with an insertion cassette (orange) to enable split site scanning, or treated with exonuclease to remove random lengths of sequence (pale green) then blunted and self-ligated to carry out deletion scanning.

The enzyme β-lactamase was chosen as an initial target for developing and validating the variable-length deletion scanning methodology. This enzyme is well studied and understood, and importantly, it confers resistant to the antibiotic ampicillin, so host resistance to ampicillin can be used as a convenient readout for β-lactamase activity. Libraries carrying deletions of between three and approximately 40 base pairs were generated and screened for β-lactamase activity. As expected, it was found that shorter deletions were more readily tolerated than longer deletions, with the longest active deletion isolated being 15 base pairs. The deletions were distributed around the structure of the enzyme (see Fig. 2), and surprisingly were not restricted to loops, but in fact were found to be approximately evenly split between α–helices and loops. The deletion library was also screened against a second generation penicillin to test for gain of extended spectrum catalytic activity, but no gain-of-function mutants could be found. A comparison of the sites tolerating deletions and

known mutational hotspots identified from clinical isolates revealed little overlap, suggesting that this deletion scanning method offers complementary mutants to those found using traditional point mutation-based methods.

In conjunction with an undergraduate project student, β-Glucuronidase was also subjected to variable-length deletion scanning, and, in contrast to β-lactamase was found to be surprisingly intolerant to deletions, accepting only a small number of single codon deletions.

b) protein split site scanning

β-lactamase was selected as a target enzyme for this project due to its convenience as a model enzyme. Library generation was carried out for two variants of β-lactamase using an insertion cassette that produces chimeric fusion proteins with FRB and FKBP. This system has the benefit of being switchable, with FRB and FKBP interacting with high affinity in the presence of the small molecule rapamycin, and not interacting in the absence of rapamycin. This is important as it allows screening and selection of candidates with the desired properties without the need for further molecular biological manipulation of samples. The assay system in droplets suffered from the fluorescent reaction product leaking rapidly from the droplets; however, a new assay system that avoids this problem has been identified, and this, coupled with progress in developing the microdroplet elements of the screening system (described below), will enable screening to be carried out in the near future.

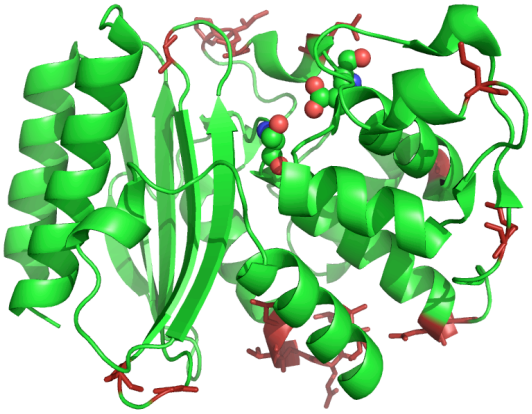

Figure 2. Tolerated deletions in β-lactamase. Structure of β-lactamase showing active site (spheres). Tolerated deletions are shown as red sticks, and can be seen to be distributed throughout the structure, including a significant proportion found in secondary structure elements.

Microdroplets work – double emulsions for sorting.

During the enzyme-focused elements of this research project it became clear that the use of microdroplets for sorting of enzyme libraries required significant improvement to be realistically and regularly useful. A droplet-based sorting platform has been developed in the lab, but it suffers from being technically challenging to use, comparatively slow (1 kHz) and prone to blockage. It was decided worthwhile to invest time and effort in developing a FACS-based system for sorting droplets, which is now established and in preparation for publication. Using FACS offers many advantages such as multiple colours and detection parameters, high throughput (up to ~ 30 kHz), widespread availability and robust instrumentation, leading to double emulsion sorting being likely to achieve significantly greater uptake than direct sorting of emulsions.

Publications:

• 10.1016/j.cbpa.2010.08.013 - Kintses, B.; van Vliet, L. D.; **Devenish, S. R.**; Hollfelder, F., Microfluidic droplets: new integrated workflows for biological experiments. *Curr Opin Chem Biol* **2010,** *14* (5), 548-55.

• 10.1039/C2LC40281E - Kaltenbach, M.; **Devenish, S. R.;** Hollfelder, F., A simple method to evaluate the biochemical compatibility of oil/surfactant mixtures for experiments in microdroplets. *Lab Chip* **2012,** *12* (20), 4185-92.

• 10.1007/978-1-62703-354-1_16 - **S. R. Devenish**, M. Kaltenbach, M. Fischlechner & F. Hollfelder Protein Nanotechnologies (Methods in Molecular Biology), Eds J. Gerrard, in the press (2013).

Patents:

• GB1211342.9 - Microfluidic Devices (Fabrice Gielen, Liisa van Vliet, Bartosz Koprowski, **Sean R. A. Devenish**, Joshua B. Edel, Xize Niu, Andrew J. deMello and Florian Hollfelder)