MULTI-FUNCTIONAL BIOCATALYSTS IN ORGANIC SYNTHESIS

THOMAS W. THORPE, JAMES R. MARSHALL and NICHOLAS J. TURNER

Department of Chemistry, University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, Manchester M1 &DN, UK.

Introduction

Enzymes display remarkable catalytic activity, evolvability and reaction promiscuity, features which together have resulted in their increased application in chemical synthesis [1]. Reaction promiscuity is manifested in several different ways and includes both the tolerance of broad substrate scope as well as the ability of enzymes to catalyse mechanistically different reactions. The latter is exemplified by the repurposing of P450 monooxygenases such that their chemistry is altered from oxygenation reactions to carbene/nitrene generation, allowing for the generation of new synthetic pathways that exploit these reactive intermediates [2]. This 'new-to-nature' chemistry of enzymes in the laboratory serves to expand the toolbox of biocatalysts available for synthetic purposes and provides new starting points for further engineering and evolution.

The ability of enzymes to catalyse two or more mechanistically distinct reactions also raises the question as to whether these different activities can simultaneously exist within a single active site. Multi-functional biocatalysts of this type would have great value in synthesis and would complement examples developed in the field of chemo-catalysis [3]. In addition, the discovery and development of a new range of multi-functional biocatalysts would have a major impact for synthetic applications, particularly in the context of cascade processes. Currently there is growing interest in the design and application of processes in which multiple-bond forming processes and functional group interconversions are achieved by combining several biocatalysts in a single vessel. For these processes, each individual biocatalyst is typically engineered separately, followed by addition to the cascade and then subsequent process optimisation. Multi-functional biocatalysts could make these approaches more efficient by reducing the enzyme count and catalyst loading, as well as streamlining enzyme engineering by minimising the sequence space that is required to be explored. The availability of multi-functional biocatalysts present different challenges that need to be considered when engineering these catalysts in order to co-evolve the different activities encoded at the active site.

The ability of a single enzyme active-site to catalyse two or more mechanistically distinct reactions requires a number of conditions to be simultaneously met, namely (i) the ability to bind structurally related substrates & products since the product from one reaction becomes the substrate for the next reaction etc. (ii) the availability of catalytic active-site residues (*e.g.* proton donors, proton acceptors, nucleophiles) that can participate in different non-bonding interactions (iii) in some cases the availability of cofactors (*e.g.* NAD(P)H, $FADH_2$) that can mediate different redox processes *e.g.* reduction of C=O as well as reduction of C=N bonds. However, these challenges are far outweighed by many of the advantages accompanied with multi-functional biocatalysts. Such enzymes essentially generate intra-enzymatic cascades, reducing overall enzyme count and loading. There are also implications in streamlining enzyme immobilisation and downstream processing.

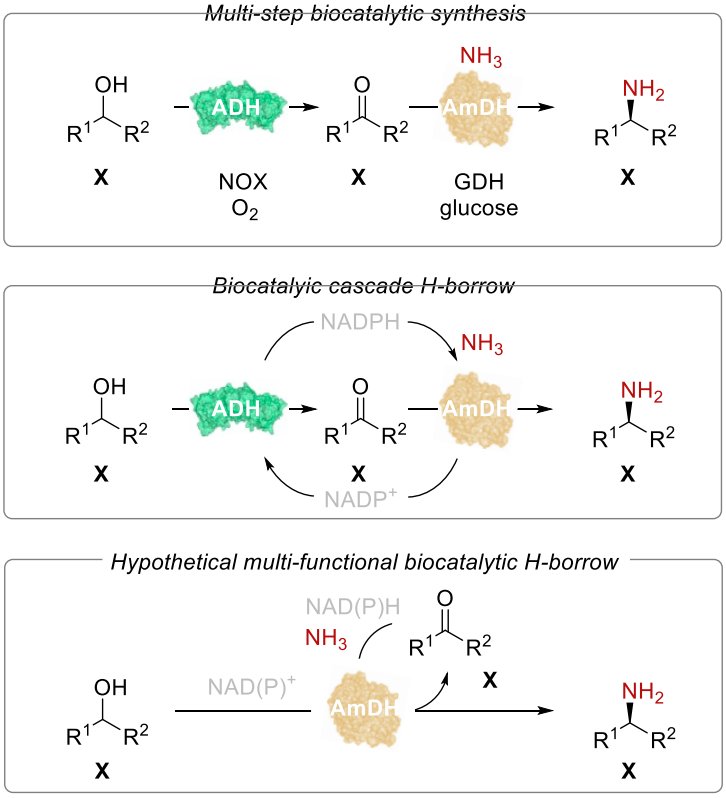

Figure 1. Advances in biocatalytic systems developed for the conversion of alcohols to amines: (i) multi-enzyme conversion of an alcohol to an amine using alcohol dehydrogenase (ADH) and amine dehydrogenase (AmDH) with cofactor recycling; (ii) replacement of the NADH/NADPH cofactor recycling modules with a shared 'hydrogen-borrowing' system; (iii) a 'hypothetical' multi-functional biocatalyst enabling a single enzyme/single cofactor system.

This discussion paper is aimed to stimulate discussion around the discovery, engineering and synthetic application of multi-functional biocatalysts. Out of the scope, although also of growing interest, are examples of multi-functional biocatalysts generated by creating an additional active-site within the protein (Plurizymes) [4] and fusion-proteins [5].

To frame the discussions examples are giving from the authors own work as set out in Figure 1 in which methods for the conversion of alcohols to chiral amines has progressed, and been simplified, by the discovery of enzymes with multiple catalytic activity.

EneIRED: A multi-functional biocatalyst that exploits iminium catalysis.

Many high value amine containing compounds have multiple stereogenic centres. Accessing these molecules and controlling their centers is a challenge as highlighted by complex biomimetic tandem catalysis and multi enzymes systems required for their synthesis. The discovery of an enzyme (EneIRED) that combines conjugate reduction and reductive amination into a single enzyme allowing exquisite control of up to 3 stereocentres generating chiral amines [6]. EneIRED, was identified from a collection of metagenomic IREDs [7] which were generated to explore the sequence space around the reductive aminase from *Aspergillus oryzae* [8]. EneIRED was initially identified by its ability to catalyse coupling of cyclohex-1-enone with allylamine to yield the fully reduced product in high conversion (Figure 2).

A comprehensive assessment of the substrate scope of EneIRED was undertaken covering enals, linear and cyclic enones, including 3-substituted cyclohexenones with largely excellent conversions to the fully saturated product. A variety of primary amines and as well as pyrrolidines could be coupled with these unsaturated carbonyls. Generally products contained 2 stereocentres, but when provided with the correct substrates, EneIRED could control 3 stereocentres with excellent selectivity, including generation of a fluorinated tertiary amine. Furthermore, EneIRED was also shown to catalyse a 6-electron conjugate reduction-reductive amination reaction starting from the di-enyl ketone.

Mechanistic studies from isotopic labelling experiments confirmed that the conjugate reduction preceded the reductive amination step (Figure 2). Structural differences in the active-site were observed in comparison to other IREDs. *i.e.* EneIRED contains an additional tyrosine residue important for the steric control of the conjugate reduction-reductive amination reactions reactions. EneIRED employs two catalytic cycles that together generate the fully reduced amine *via* two different iminium intermediates, reminiscent of autotandem organocatalytic systems (Figure 2). EneIRED exemplifies the ability of multi-functional

biocatalysts to combine different chemistries into a simple protein biomolecule whilst generating compounds of high value.

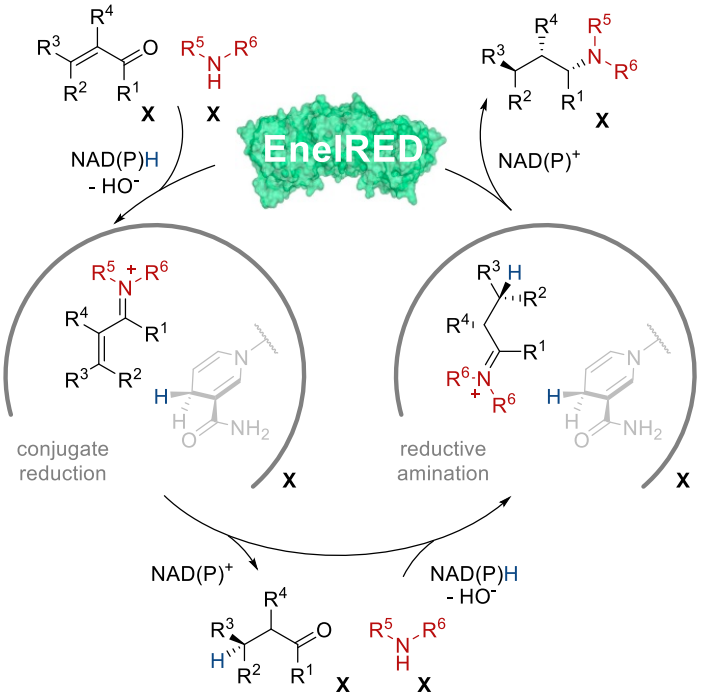

Figure 2. Catalytic cycle of the multi-functional biocatalyst EneIRED which catalyses conjugate reduction-reductive amination via iminium-type catalysis.

Summary and future perspectives

Although multi-functional biocatalysts have the potential to transform organic synthesis, the field is clearly at an early stage of development. Such enzymes meet the demand for the development of more streamlined and smarter biocatalysts [9, 10]. Coupled with advances in other areas (*e.g.* metagenomics, photobiocatalysis), enzymes possessing even more complex chemistries will emerge, thereby enabling new retrosynthetic pathways to be developed [11].

For a more sustainable future and in the current climate of the world, companies and institutions are scrutinising every aspect of their practices to reduce their carbon footprint [12]. Multi-functional enzymes can contribute to this challenge by reducing the overall enzyme loading, with a concomitant reduction in engineering of multiple central and auxiliary enzymes. Substrate

localization is another element which is improved, in turn leading to greater reaction efficiency.

Acknowledgements

We acknowledge the ERC for award of an Advanced Grant (BIO-H-BORROW).

References

1. J.B. Pyser, S. Chakrabarty, E.O. Romero, A.R.H. Narayan *ACS Cent. Sci*., **7**, 1105–1116 (2021).
2. D.C. Miller, S.V. Athavale, F.H. Arnold *Nature Synth*., **1**, 18-23 (2022).
3. N. Liu, Y.-F. Xie, C. Wang, S.-J. Li, D. Wei, M. Li, B. Dai *ACS Catal*., **11**, 9945–9957 (2018)
4. D. Carballares, R. Morellon-Sterling, R. Fernandez-Lafuente *Int. J. Mol. Sci*., **23**, 5304 (2022).
5. F.S. Aalbers, M.W Fraaije *ChemBioChem*, **20**, 20-28 (2019).
6. T.W. Thorpe, J.R. Marshall *et al*., *Nature* **604**, 86–91 (2022).
7. J.R. Marshall, P. Yao *et al*., *Nature Chem.,* **13**, 140-148 (2021).
8. G.A. Aleku, S.P. France *et al*., *Nature Chem.,* **9**, 961–969 (2017)
9. K.F. Biegasiewicz, S.J. Cooper, X. Gao, D.G. Oblinsky, J.H. Kim, S.E. Garfinkle, L.A. Joyce, B.A. Sandoval, G.D. Scholes, T.K. Hyster *Science*, **364**, 1166–1169 (2019)
10. R.A. Sheldon, D. Brady *ACS Sustain. Chem. Eng.,* **9**, 8032–8052 (2021)
11. N.J. Turner, E. O'Reilly *Nature Chem. Biol.,* **9**, 285–288 (2013); W. Finnigan, L.J. Hepworth, S.L. Flitsch, N. J. Turner *Nature Catal*., **4** 98–104 (2021).
12. M. Okereke *J. Climate Change & Health,* **4**, 100049 (2021).

Keywords: Biocatalysis; Multi-Functional Catalysts; Sustainability; Chiral Amines; Directed Evolution; Protein Engineering: Pharmaceuticals; Cofactors; Active-Site; Fusion-Proteins.