# Enabling the chemistry of life

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**Enzymes are the subset of proteins that catalyse the chemistry of life, transforming both macromolecular substrates and small molecules. The precise three-dimensional architecture of enzymes permits almost unerring selectivity in physical and chemical steps to impose remarkable rate accelerations and specificity in product-determining reactions. Many enzymes are members of families that carry out related chemical transformations and offer opportunities for directed *in vitro* evolution, to tailor catalytic properties to particular functions.**

The myriad chemical transformations carried out by every living organism are enabled by hundreds to thousands of proteins (enzymes) and, less frequently, RNAs (ribozymes), which have catalytic activity for conversion of a particular set of substrates to specific products. Some of these reactions are carried out by related families of protein biocatalysts, which act generically in the same way but exert specific recognition for transformation of a particular substrate molecule. For example, the orderly control of the location and lifetime of proteins in cells is managed by dozens of related proteases that hydrolyse peptide bonds of protein substrates in ways that are controlled in time and space. Proteases can be exquisitely specific for a particular peptide bond in a protein substrate, or they can be relentlessly nonspecific: the former set of proteases are involved in turning on biological signals, the latter in the clean-up phases of degradation and protein turnover.
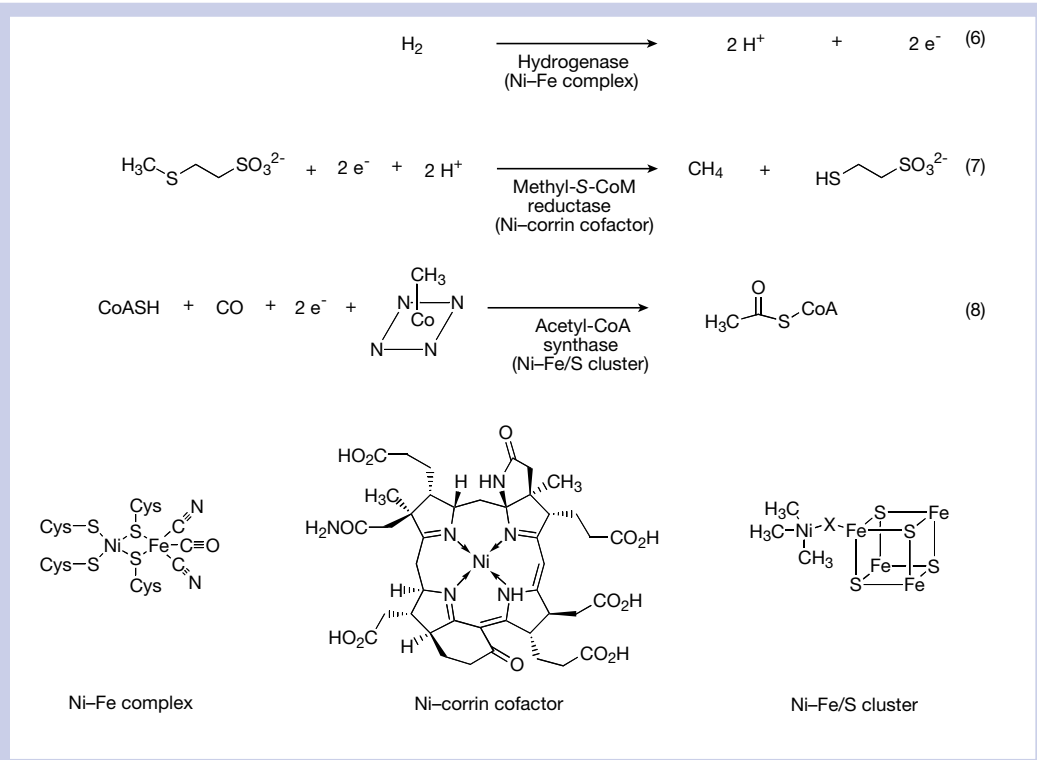
When cells respond to external messenger molecules, such as the protein growth factors and hormones erythropoietin and insulin, or small-molecule hormones such as adrenaline or prostaglandins, signalling pathways are set in motion by catalytic action of cascades of protein kinases. The protein kinases are built from a small set of architectural types, and all catalyse phosphoryl transfer from ATP to the side-chain hydroxyl of serine, threonine or tyrosine residues. There are hundreds of such kinases in animal genomes. Selectivity is imposed on this generic chemical phosphorylation reaction by protein–protein interactions between a given kinase and its protein substrate and by cascades of such kinase/protein substrate pairs that ultimately lead to changes in activity and location of proteins, and to selective gene activation.

In addition to the large number of enzymes that act on macromolecular protein substrates, there are also enzymes that engage in truly sophisticated chemistry on small



**Figure 1** Diverse chemical reactions facilitated by biocatalysts. ACC lyase, 1-aminocyclopropane-1-carboxylate lyase; IPNS, isopenicillin *N* synthase; OMP decarboxylase, orotidine-5′-phosphate decarboxylase.

$$k_{cat}/k_{uncat} = 10^{23}/1$$
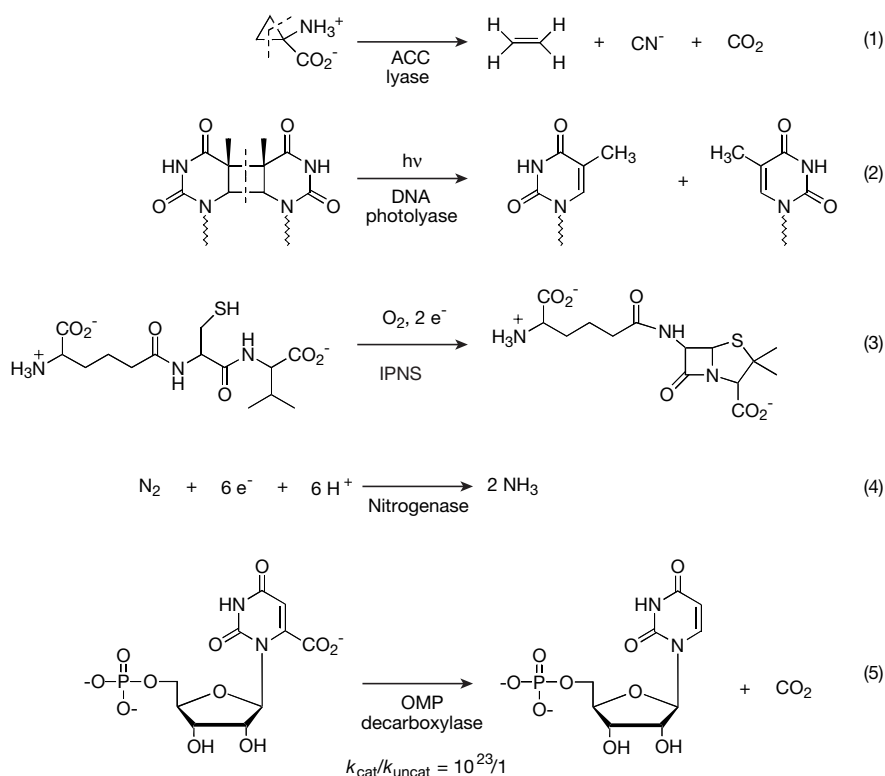
**Figure 2** Nickel-based enzymatic transformations in methanogenic archaebacteria.

$$H_2 \xrightarrow[\text{(Ni–Fe complex)}]{\text{Hydrogenase}} 2\,H^+ \;+\; 2\,e^- \qquad (6)$$

$$H_3C{-}S{-}CH_2CH_2{-}SO_3^{2-} \;+\; 2\,e^- \;+\; 2\,H^+ \xrightarrow[\substack{\text{reductase}\\ \text{(Ni–corrin cofactor)}}]{\text{Methyl-}S\text{-CoM}} CH_4 \;+\; HS{-}CH_2CH_2{-}SO_3^{2-} \qquad (7)$$

$$CoASH \;+\; CO \;+\; 2\,e^- \;+\; [\text{Co corrin (CH}_3)] \xrightarrow[\substack{\text{synthase}\\ \text{(Ni–Fe/S cluster)}}]{\text{Acetyl-CoA}} H_3C{-}C(O){-}S{-}CoA \qquad (8)$$

Ni–Fe complex

Ni–corrin cofactor

Ni–Fe/S cluster

organic molecules. The fragmentation of 1-aminocyclopropane-1-carboxylate to the fruit-ripening hormone ethylene[1], the photon-induced $2+2$ cycloreversion of thymine dimers to repair DNA damaged by ultraviolet light[2], the bis-cyclization of the tripeptide aminoadipoyl-cysteinyl-D-valine (ACV) to isopenicillin N (ref. 3), and the reduction of dinitrogen ($N_2$) to two molecules of ammonia ($NH_3$) during nitrogen fixation[4] are just a few examples of the range of biological chemistry facilitated by biocatalysts (Fig. 1). Enzymes as biocatalysts are remarkable not only in themselves, but also for the inspiration and guidance they provide to synthetic organic and inorganic chemists striving to reproduce and expand nature's chemical repertoire. Several of the useful attributes of biocatalysts, such as their use as reagents for chemical synthesis and scale-up, and directed evolution to tailor chemical transformations, are explored in other articles in this Insight.

## Biocatalysts and their *ex vivo* utility

Biocatalysts carry out the chemistry of life, the controlled chemical transformations in primary metabolism and the generation of natural-product diversity in secondary metabolism of plants and microbes. Classically, the subset of proteins with catalytic activity — the enzymes — has been the focus of biocatalysis research. But there is an increasing focus on catalytic RNA (ribozymes), the discovery of which in the 1980s supported the arguments for an 'RNA world'[5,6] antecedent to the contemporary world where proteins are the work-horse biocatalysts. Most recently, Joyce and co-workers[7] have reported catalytic DNA molecules, and directed evolution of both RNA and DNA biocatalysts will continue to expand their potential. The current set of RNA and DNA catalysts have been assayed and developed for activities in nucleic-acid replication and in protein synthesis[8,9], but it remains to be seen how suitable they will be for the chemically diverse reactions encompassed by existing enzyme catalysts.

The twin hallmarks of enzyme biocatalysts are the remarkable specificities and sometimes phenomenal rate accelerations achieved. A typical enzyme, with a relative molecular mass of 50,000 ($M_r$ 50K), is comprised of 450 amino-acid residues: 19 chiral L-amino acids and glycine. If glycine makes up 10% of the residues, then there are at least 400 residues with chiral centres to provide an asymmetric microenvironment for substrate binding and subsequent chemical

transformation in the enzyme's active site. This is the underlying structural basis for the action of all enzymes as chemoselective and regio- and stereospecific catalysts. In terms of rate accelerations, the relative values over nonenzymatic rates of transformation are often $10^{10}$, for example for protease-mediated hydrolysis of peptide bonds, and can reach $10^{23}$ in the example of orotidine decarboxylase in the pyrimidine biosynthetic pathway[10] (reaction 5 in Fig. 1). In absolute terms, enzymes have turnover numbers from as slow as one catalytic event per minute to $10^5$ per second (as in the hydration of $CO_2$ to $HCO_3^-$ by carbonic anhydrase)[11].

These two attributes of enzymatic biocatalysts have spurred much investigation into both the structural and mechanistic bases of the chemical transformations and have stimulated much of the study of enzymes in chemical synthesis (see review in this issue by Koeller and Wong, pages 232–240). *In vivo*, enzymes operate in buffered aqueous environments with ionic strength and pH control, although microbes that live at extremes of temperature and pH are of particular current interest because of the stability of their constituent enzymes. Much attention in biocatalyst process design (see accompanying review by Witholt et al., pages 258–268) is on how to prolong useful lifetimes of enzyme catalysts and to have them operate in media not ordinarily compatible with life.

The past two decades have also witnessed an intense exploration of catalytic antibodies[12]. To prepare these antibodies, ligands are synthesized that typically mimic transition states of particular chemical transformations, such as ester hydrolysis, amide synthetase and Claisen condensation. Monoclonal antibodies are then selected that display high-affinity binding to the ligands, thus enriching for antibody proteins with a binding-site geometry complementary to the shape of the true transition state. Some of the antibodies selected in this way show catalysis of the desired reactions, with the selectivity and rate accelerations expected for chiral protein-based catalysts[13,14]. But low catalytic turnover numbers have so far limited the use of catalytic antibodies in chemical synthesis or process work.

## Biocatalysts or biomimetic catalysts?

With their unerring stereoselectivity and high catalytic efficiency, nature's enzymatic catalysts have been a stimulus and counterpoint

**Figure 3** Cyclization catalysed by the thioesterase domain of tyrocidine synthetase.
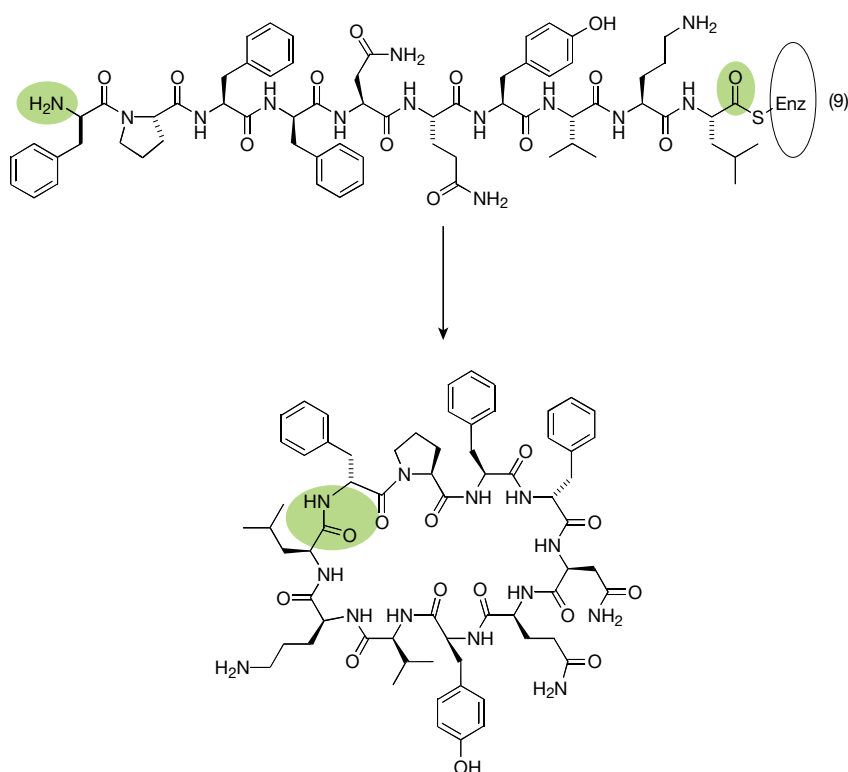
for generations of chemists who have designed and tested bioorganic and bioinorganic versions of biomimetic catalysts, whether for example to mimic macrocyclizations of natural products or to produce analogues of hydrogenase or nitrogenase catalysts or the photosynthetic splitting of water[15]. The mimics may operate under harsher solvent and temperature conditions, and may be more robust in terms of lifetime (if not throughput per catalyst molecule). When organic coenzymes (such as flavins, pyridoxal or thiamin) or inorganic cofactors (iron/sulphur clusters, metalloporphyrins) are crucial components of the enzymatic catalysis, the biomimetic and natural catalysts often show design convergence and may recapitulate some of the steps in biocatalyst evolution. The three nickel enzymes in methanogenic bacteria (thought to be contemporary descendants of primordial organisms), which carry out nickel-based hydrogenation, nickel-based methyl thioether reduction to methane, and nickel-based carbonylation of a methyl co-substrate to produce acetate, can be viewed as such an intersection[16,17] (Fig. 2).

When is it worthwhile for the synthetic or process chemists to reject synthetic reagents and catalysts in favour of enzymes to carry out a specific transformation? This may vary with individual preference and each case must be judged on its own merits. Lipases and other hydrolases have clear advantages in kinetic resolutions of intermediates (see below), penicillin acylases have long been a mainstay of semisynthetic processes in the β-lactam antibiotic industry, and enzymatic aldol condensations have shown their worth in complex oligosaccharide syntheses[18].

## Chemical transformations well suited to enzymes

The accompanying review by Khosla and Harbury (pages 247–252) explores the multimodular enzymes that function as molecular solid-state assembly lines for the generation of thousands of polyketide natural products and non-ribosomal peptide antibiotics, including important medicinal compounds such as erythromycin, rapamycin, epothilone, lovastatin, penicillins, cyclosporin and vancomycin[19–21]. These sequentially elongating acyl transfers seem particularly apt loci for use as enzymatic rather than biomimetic catalysis. Some of the assembly lines, such as those for erythromycin or cyclosporin, produce the intramolecularly cyclized macrolactones or macrolactams. It has recently been shown[22] that the last 30K (thioesterase) domain of the 724K protein assembly line of tyrocidine synthetase retains the ability to cyclize 9–11-residue peptidyl thioesters with regio- and stereoselectivity, raising the prospect for practical enzymatic macrocyclizations by a robust, small protein fragment (Fig. 3, reaction 9).

The reprogramming of the component enzyme domains of these assembly lines to create new, unnatural 'natural' products is one of the goals of combinatorial biosynthesis. The order of the enzymatic domains in the assembly lines specifies which monomer substrates are activated, condensed and elongated. So altering the order and permutations of these domains offers the chance to control product structure. The directed evolution of the catalytic domains of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) assembly lines by gene shuffling and other approaches (see accompanying review by Arnold, pages 253–257) can create designed diversity in complex natural products.

Once the nascent products have been released from the PKS and NRPS assembly lines, the polyketide or polypeptide may require further enzymatic transformations to attain antibiotic properties. This is the case for penicillins, vancomycin and erythromycin, to cite just three important examples[19]. Baldwin and co-workers[23,24] showed that the tripeptide ACV is oxidatively transformed to the 4-5 bicyclic β-lactam ring system by isopenicillin N synthase (IPNS; Fig. 1, reaction 3). IPNS is a member of a substantial family of iron-containing enzymes that use $Fe^{2+}$ to activate both $O_2$ and the specific co-substrate for complex redox chemistry[25]. In IPNS, both atoms of dioxygen are reduced to water and the ACV tripeptide undergoes four-electron oxidation and directed C–S bond and C–C bond formation as the β-lactam forms. A cousin of IPNS, the expandase enzyme, is used by cephalosporin-producing organisms to expand the five-membered ring in penicillins to the six-membered ring in cephalosporin

**Figure 4** Comparison of expandase active site with a typical haemprotein oxygenase.

Expandase active Site

P450 haem active Site

$$R\text{-}[\text{}]\text{-}S + O_2 + 2e^- \xrightarrow{\text{Expandase (Fe)}} R\text{-}[\text{}] \quad (10)$$
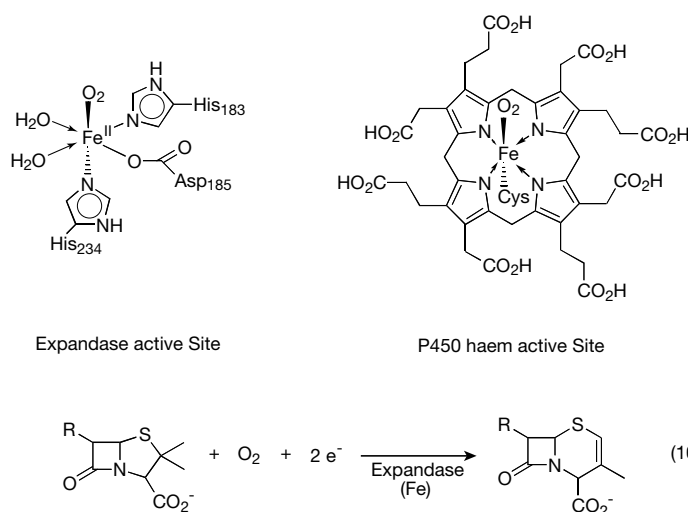
antibiotics (Fig. 4, reaction 10). The ligand set around the active-site iron — one Glu, two His residues — is the same, but the reaction flux is distinct (Fig. 4). Other members of this non-haem dioxygenase family include the enzyme responsible for hydroxylating prolyl residues in protocollagen to predispose it to triple-helix formation in mature collagen, the most abundant protein in the human body. There are clear potential benefits to understanding the molecular basis for how the high-valent oxo-iron reagents are controlled and directed to flawlessly different chemical outcomes in the members of this redox enzyme family, so that they might be subjected to *in vitro* evolution to generate new reaction fluxes.
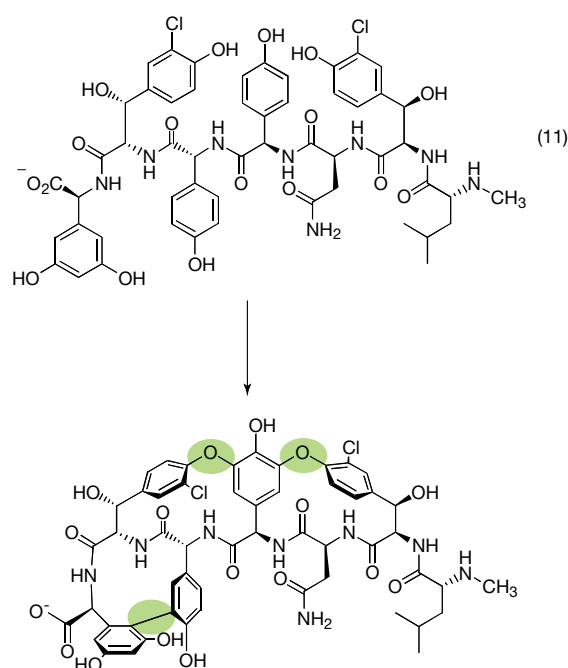
Many natural products, from morphine and codeine to vancomycin, undergo oxidative cyclization reactions that are regio- and stereospecific and seem to be mediated by a different superfamily of iron-containing oxidases, the cytochromes P450, with $Fe^{2+}$ embedded in a haem macrocycle (Fig, 4). Protein superfamilies are groups of proteins with distinct chemical functions, amino-acid sequences of recognizable but sometimes marginal homology, and convergent three-dimensional structures. In the vancomycin family of glycopeptide antibiotics there are three crosslinks that convert an acyclic heptapeptide, the product of the NRPS assembly line, into a rigid scaffold, crosslinked at $Tyr_2$-$PheGly_4$-$Tyr_6$ and $PheGly_5$-$dihydroxyPheGly_7$ (Fig. 5, reaction 11). There are three P450 cytochromes in the biosynthetic gene cluster; each might enact a regiospecific phenolic crosslink. Harnessing such catalysts for related transformations might lead to new vancomycins.

Several natural products contain tandem five-membered-ring heterocycles (oxazoles and thiazoles) that arise from enzymatic cyclization of serine or cysteine residues in peptide precursors[26]. These include the *Escherichia coli* antibiotic microcin B17, which kills neighbouring bacteria by poisoning the enzyme DNA gyrase and thus blocking DNA replication, in much the same way as does the best-selling antibiotic ciprofloxacin[27] (Fig. 6). Such heterocycles are also found in the iron-chelating siderophores that act as virulence factors in infections by *Pseudomonas aeruginosa*, *Vibrio cholerae* and the causative agent of the black plague, *Yersinia pestis*[28,29]. Enzymes that heterocyclize serine, threonine and cysteine side chains in peptides (Fig. 6, reactions 12, 13) may create either DNA-seeking or iron-chelating sites in any peptide library that could then be screened for biological activity.

## Superfamilies, genomics and enzyme evolution

The iron-containing dioxygenases that include IPNS and expandase, and the cytochrome P450 variants that introduce crosslinks, comprise redox enzyme superfamilies that are good candidates for

engineering for altered catalytic properties and specificities. Genomic and proteomic searches can identify many enzyme superfamily members through amino-acid sequence homologies, in which scaffolding and structural architecture will be predictable. Some of these proteins are of unknown ('orphan') function, and the assignment of function is one of the major postgenomic challenges of proteomic research. Recent cases in the crotonase superfamily (Fig. 7, reactions 14–16) and enolase superfamily (Fig. 7, reactions 17–19)[30–32] indicate that the active sites all generate carbanionic transition states from bound substrates and then use carbanion chemistry for directed fluxes and distinct product outcomes. These families should be fruitful starting points for directed enzyme evolution to elicit new fluxes, based on the knowledge that carbanion chemistry will be facilitated

(11)

**Figure 5** Crosslinking by cytochrome P450 enzymes to produce the vancomycin Aglycone.

**Figure 6** DNA gyrase inhibitors and biosynthesis of peptide heterocycles.



**Figure 6** DNA gyrase inhibitors and biosynthesis of peptide heterocycles.

in one of the co-substrates and that binding sites can be re-engineered for electrophilic substrate components.

Once an enzyme has been evolved to have a detectable and desirable new activity[33], additional rounds of *in vitro* evolution can improve its stability and robustness. The biological selection methods are sufficiently powerful that one can find outcomes that are very rare biologically in a short space of time. A good example is the recent report[34] of expression of a functional carotenoid biosynthetic pathway in *E. coli* by selecting for bacteria that become red. The continuing progress in biological production of polyhydroxyalkanoate polymers with controlled sizes and properties[35] by engineering the respective polymerases increases the likelihood of economically viable production of these biodegradable plastics by biocatalysis.

## Enzymes in bioremediation

One of the most active areas of applied enzymology in the past two decades has been the study of enzymes capable of bioremediation: the breakdown of organic and inorganic pollutants. There are now substantial databases of enzymes and the bioremedial transforma-

tions[36] they catalyse, which include the breakdown of aromatic and heteroaromatic pollutants by oxidative, reductive and hydrolytic transformations. Iron-containing dioxygenases and monooxygenases, with overlapping regio- and chemospecificities, are superfamilies that represent good starting points for application of many of the strategies noted here and in the specific accompanying articles for directed enzyme evolution to broaden substrate recognition. It is likely that bioremediation scenarios in the field will require the tailored enzymes to work in their host microbial cells rather than as *ex vivo* catalysts. Engineering of multistep metabolic pathways by introducing heterologous genes[37] and *in vivo* expression may well be required for efficient degradation of non-biogenic compounds. As many waste sites have a witches' brew of foreign compounds, multiple pathways engineered stably into a microbe or, more probably, mixed bacterial communities that can coexist stably, will be required. The enzymology of processing of toxic inorganic ions has also progressed in recent years to include mercury, copper, cadmium, silver, arsenic and cobalt. This might ultimately make remediation schemes for inorganic pollutants feasible[38].

**Figure 7** Representative reactions catalysed by the crotonase superfamily and the enolase superfamily.



**Figure 7** Representative reactions catalysed by the crotonase superfamily and the enolase superfamily.

## Conclusions

As structural genomics continues to reveal the folds and scaffolds of several members of all the principal superfamilies of enzymes, the molecular bases of recognition of substrates and directed fluxes through specific transition states to particular subsets of products will become increasingly clarified. In turn this will aid in enzyme evolution to select and detect new activities and then to incorporate improved catalytic efficiency, attributes of specificity, and structural features optimized to a given operating microenvironment. For both *in vitro* applications for a specific synthetic chemical step and for *in vivo* construction of new metabolic pathways, the applications for enzymes in practical biocatalysis will continue to burgeon. Small-molecule chemical transformation catalysed by enzymes from microorganisms that live in unusual environments or conduct chemical warfare against their neighbours have been and are likely to remain good hunting grounds for new enzyme transformations. □

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