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Hybrid enzymes: manipulating enzyme design

Andrew E. Nixon, Marc Ostermeier and Stephen J. Benkovic

Hybrid enzymes are engineered to contain elements of two or more enzymes. Hybrid-enzyme approaches, by taking advantage of the vast array of enzymatic properties that nature has evolved, as well as the strategies that nature has used to evolve them, are becoming an increasingly important avenue for obtaining novel enzymes with desired activities and properties.

The primary goal of protein engineering is to create a novel protein Y that possesses some improved or novel property by changing the amino acid sequence of an existing protein X. There are many reasons why one might want to create protein Y: protein X may exhibit poor stability or low levels of catalysis under industrially relevant conditions, such as in organic solvents or at extremes of temperature or pH; the production of protein X in recombinant hosts may suffer from inefficient folding and protease susceptibility; the catalytic functions of protein X may be altered or improved, or its substrate specificity broadened or narrowed; and, finally, one may wish to create a protein Y with novel reactivity. However, even though a great deal of information on structure–function relationships is available and a myriad of genetic-engineering techniques enables the creation of novel proteins, the conversion of protein X into protein Y remains a difficult task[1].

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Although many examples of catalysts created using catalytic-antibody methodology have been described, the predominant approach to biocatalyst design has focused on the modification of existing enzymes. Increasingly, groups are using an approach that couples random mutagenesis with a suitable method of selecting enzymes with altered properties. A less stochastic approach to protein engineering is to make use of properties in existing enzymes and, guided by the detailed sequence and structural knowledge currently available, construct chimeric or hybrid enzymes.

The term 'hybrid enzyme' is a rather nebulous term that warrants further definition. For the purposes of this article, a hybrid enzyme is considered to be composed of elements of more than one enzyme. Thus, hybrid enzymes

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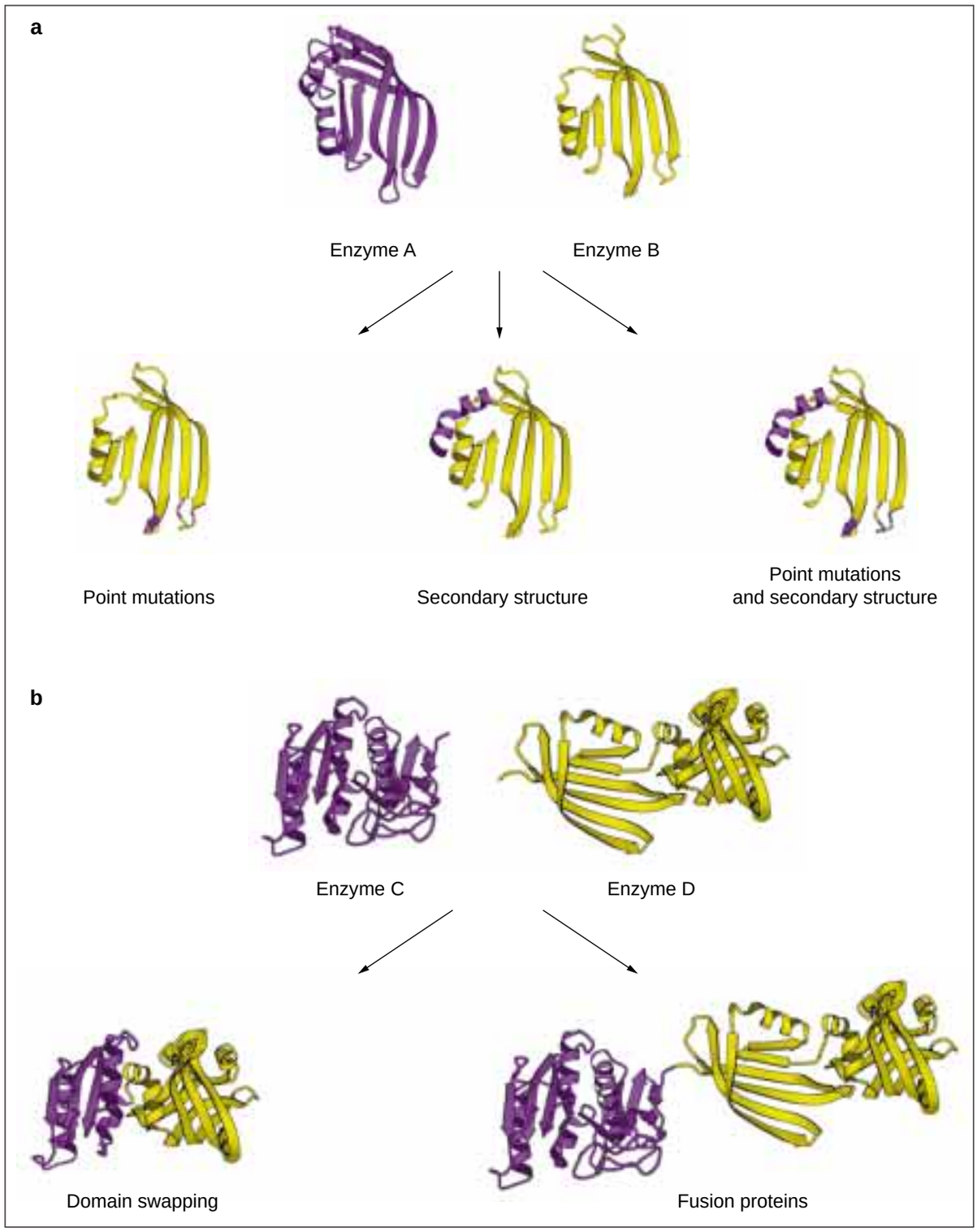

Figure 1

Generation of hybrid enzymes. (**a**) Substitution of point mutations, secondary structures or both from enzyme A into a homologous enzyme B. (**b**) Exchange of functional domains between enzymes C and D or fusion of the intact enzymes.

can be generated in a number of ways (Fig. 1): an existing enzyme can be altered by a single point mutation (or series of point mutations) based on structures existing in a second enzyme; similarly, secondary-structural elements or whole domains of enzymes, or monomeric units of multimeric enzymes, can be exchanged; fusions between two enzymes that have separate and distinct activities are also, by this definition, hybrid enzymes.

The construction of hybrid enzymes parallels the strategies that nature uses to evolve enzymes. It is generally thought that enzymes have evolved to fit a specific niche in biology through such processes as gene duplication, domain recruitment and fixation of multiple point mutations. Similarly, hybrid-enzyme approaches seek to recruit established functions and properties from existing enzymes and incorporate them

into the engineered enzyme. These techniques have been shown to be useful in the alteration of nonenzymatic as well as enzymatic properties and also as tools for understanding structure–function relationships. In addition, the creation of hybrid enzymes can expand the potential uses of natural enzymes. Enzymes, or fragments of enzymes, could potentially serve as building blocks for proteins capable of catalysing reactions not observed in nature.

Alteration of noncatalytic properties

Early demonstrations of the functionality of interspecies hybrids[2,3] have led to many studies on hybrids between highly homologous enzymes in order to confer some nonenzymatic property, such as thermostability[4,5], of one enzyme on the other. These hybrids are created by the exchange of residues or structures between homologous regions of related enzymes. Such exchanges have generally resulted in hybrid enzymes with properties intermediate between those of the two parent enzymes. For example, hybrids of an *Agrobacterium tumefaciens* β-glucosidase (optimum activity at pH 7.2–7.4 and 60°C) and a *Cellvibrio gilvus* β-glucosidase (optimum activity at pH 6.2–6.4 and 35°C) resulted in hybrids that were optimally active at pH 6.6–7.0 and 45–50°C, and possessed K_m values for various saccharides that were intermediate between those of the parent enzymes[6].

Regulation of activity can be engineered into an enzyme through the insertion of a short peptide loop of defined function. This has been shown by the insertion of a protease-binding sequence into interleukin-1β (IL-1β), a protein that is insensitive to proteases, to create an elastase- and chymotrypsin-sensitive IL-1β[7]. The insertion of epitopes in the vicinity of an enzyme's active site can create hybrids whose activity can be modulated by antibodies specific for that epitope[8]. It is also feasible that the creation of hybrid enzymes could improve the folding and production of recombinant proteins.

Often, the exchange of homologous regions between related enzymes results in hybrid enzymes with diminished activity. Not too surprisingly, the lower the similarity, the more likely that the hybrid will have diminished or no activity. However, random mutagenesis can be used to restore the enzyme's activity, presumably by restoring proper interactions for folding, stability and obtaining the correct structural formation. For example, RTEM-1 β-lactamase and a β-lactamase from *Proteus vulgaris* have 37% similarity; of a series of 18 hybrids between the two, most were inactive, with a few hybrids with partial or trace levels of activity[9]. However, random mutagenesis of some of the hybrids with partial or trace activity dramatically improved their activity, even though the residues mutated did not interact with the substrate.

Creation of enzymes with novel activities

One of the most powerful uses of hybrid enzymes is the creation of enzymes with novel catalytic activities. The term 'retrofitting' is used in biocatalyst design to describe the process of introducing elements into an existing protein to modify its functionality[10]. This can be looked at in terms of how the changes are made (by point mutation, exchange of secondary structure or exchange of domains, as previously discussed[11]) or from the view of 'what is the engineering goal?' The latter approach can conveniently be divided into three levels: (1) the modulation of specificity or catalysis in existing enzymes; (2) the introduction of catalytic residues into a binding protein; and (3) the introduction of catalytic and binding properties into a protein scaffold. The kinetic parameters of several of the hybrid enzymes discussed below are shown in Table 1.

Modulation of specificity or catalysis in existing enzymes

This forms by far the largest group of hybrid enzymes as it is conceptually the easiest way to create enzymes with novel properties. In fact, even single amino acid changes have been shown to be sufficient to convert catalytic activities, as in the conversion of a glutaconate-CoA transferase from *Acidaminococcus fermentans* into an acyl-CoA hydrolase[20]. However, it has generally been enzyme specificity that has been modulated, either by a series of point mutations or by the exchange of secondary structures or domains.

Point mutations and secondary structure

Proteases have been a common target for exchanging substrate specificity: the substrate specificity of the subtilisin from *Bacillus licheniformis* was incorporated into the subtilisin BPN′ from *Bacillus amyloliquefaciens* by the exchange of three residues[16]; subtilisin BPN′ was mutated to cleave dibasic[17] or tribasic[18] substrates by comparing its sequence to those of eukaryotic homologs known to cleave dibasic or tribasic substrates and executing point mutations; trypsin was converted into chymotrypsin by the exchange of four residues in the active site and the exchange of two nonstructural surface loops[12].

Exchanging residues in the coenzyme-binding domains of glutathione reductase[14] and lipoamide dehydrogenase[21] successfully converted their cofactor preferences from NADP to NAD and NAD to NADP, respectively. The conversion of an NAD preference to NADP in *Thermus thermophilus* isopropylmalate dehydrogenase required more than a few point mutations[22]: a 13-residue α helix from the NADP-utilizing *Escherichia coli* isocitrate dehydrogenase (with four substitutions suggested by structural modeling to prevent steric problems) was used to replace a β turn. Four additional binding-pocket mutations resulted in a shift in preference to NADP by a factor of 10^5.

A similar dramatic change in substrate specificity, determined by comparisons of the apparent second order rate constant (or specificity constant) k_{cat}/K_m[23], has been observed for an engineered variant of the thermostable lactate dehydrogenase from *Bacillus stearothermophilus*. A single amino acid substitution, suggested by sequence and structural comparisons with a thermostable malate dehydrogenase, resulted in a 10^3 increase in k_{cat}/K_m for oxaloacetate and a 10^4 decrease in k_{cat}/K_m for pyruvate; this led to a 10^7 overall change in specificity[15] (Table 1). Using detailed knowledge of the enzyme's structure and mechanism, it has been possible to extend this, first to broaden the enzyme's specificity[24] and then to change the specificity from pyruvate to phenylpyruvate[25].

Table 1. Kinetic parameters of selected hybrid enzymes

Enzyme	Desired conversion	Enzyme assayed (substrate or cofactor)	Kinetic parameters			Change in k_{cat}/K_m (ratio of activities of hybrid and wild type)[j]	Refs
			K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)		
Trypsin[a]	Convert to chymotrypsin	Trypsin	650	30	4.6×10^4		12
		Chymotrypsin	50	33	6.0×10^5		
		Hybrid	27	33	1.2×10^6	26	
Chymotrypsin[b]	Convert to trypsin	Chymotrypsin	110	7.2	6.9×10^4		13
		Trypsin	1.3	1.1×10^4	8.4×10^9		
		Hybrid	1100	75	1.3×10^4	0.19	
Glutathione reductase[c]	Convert cofactor specificity from NADPH to NADH	Glutathione reductase (NADPH)	22	267	1.2×10^7		14
		Hybrid (NADPH)	220	11	5.0×10^4	4.2×10^{-3}	
		Glutathione reductase (NADH)	2000	11.3	5.7×10^3		
		Hybrid (NADH)	86	35	4.1×10^5	72	
Lactate dehydrogenase[d]	Convert substrate specificity from pyruvate to oxaloacetate	Lactate dehydrogenase (oxaloacetate)	1500	6	4.0×10^3		15
		Hybrid (oxaloacetate)	60	250	4.2×10^6	1050	
		Lactate dehydrogenase (pyruvate)	60	250	4.2×10^6		
		Hybrid (pyruvate)	1800	0.9	5.0×10^2	1.2×10^{-4}	
Subtilisin BPN′[e]	Convert substrate specificity to that of *Bacillus licheniformis* subtilisin	Subtilisin BPN′	1.4×10^5	50	360		16
		Subtilisin from *B. licheniformis*	2.0×10^5	510	2500	7.2	
		Hybrid	9.4×10^4	250	2600		
Subtilisin BPN′[f]	Convert substrate specificity from hydrophobic sequences to dibasic or tribasic sequences	Subtilisin BPN′ (hydrophobic)	110	29	2.6×10^5		17,18
		Kexilisin hybrid (hydrophobic)	1800	3.4	1.9×10^3	7.3×10^{-3}	
		Furilisin hybrid (hydrophobic)	ND[h]	ND[h]	8.0[h]	3.1×10^{-5}	
		Subtilisin BPN′ (dibasic)	1700	2.8	1.7×10^3		
		Kexilisin hybrid (dibasic)	41	15	3.7×10^5	220	
		Subtilisin BPN′ (tribasic)	ND[i]	ND[i]	ND[i]		
		Furilisin hybrid (tribasic)	29	9.8	3.4×10^5		
fH_4F-hydrolase[g]	Construct GAR transformylase by domain recruitment	fH_4F-hydrolase (fDDF)	7	2.6×10^{-2}	3.7×10^4		19
		GAR transformylase (fDDF/GAR)	17/19	16	9.4×10^5/ 8.4×10^5		
		Hybrid (fDDF/GAR)	35/16	4×10^{-4}	11/25	>400[j]	

[a]Succinyl-AAPF-thiobenzyl ester as substrate; pH 6.5, 37°C
[b]Succinyl-AAPR-amino-4-methylcoumarin as substrate, pH 8.0, 37°C
[c]Oxaloacetate as substrate; pH 4.7 (wild type), pH 5.4 (mutant), 30°C
[d]pH 8.6, 25°C
[e]Subtilisin BPN′, subtilisin from *Bacillus amyloliquefaciens*; succinyl-AAPF-*p*-nitroanilide as substrate; pH 6.0, 25°C
[f]Kexilisin, hybrid subtilisin BPN′ designed to cleave dibasic sequences; furilisin, hybrid subtilisin BPN′ designed to cleave tribasic sequences; hydrophobic substrate, succinyl-AAPF-*p*-nitroanilide; dibasic substrate, succinyl-AAKR-*p*-nitroanilide; tribasic substrate, succinyl-KAKR-*p*-nitroanilide pH 8.2, 25°C
[g]fH_4F, N^{10}-formyltetrahydrofolate; GAR, glycinamide ribonucleotide; fDDF, formyl-dideazafolate; pH 7.5, 30°C
[h]Unable to saturate the enzyme; apparent k_{cat}/K_m calculated from rates at low substrate concentrations assuming $v = (k_{cat}/K_m)[E][S]$, where [E] and [S] are the concentrations of the enzyme and substrate, respectively.
[i]Cleavage of substrate so poor that assaying was impossible owing to predominant cleavage between succinyl-KAK and R-*p*-nitroanilide
[j]Change with regards to fH_4F-hydrolase cannot be determined, because fH_4F-hydrolase has no measurable GAR-transformylase activity. Based on the detection limit, the ratio of the k_{cat}s is >400. Fold change with regards to GAR transformylase is 3.0×10^{-5}.
Abbreviations: AAPF, AAPR, four-amino-acid linkers; ND, not determined.

Functional domains

Whereas the above studies used a series of point mutations and/or exchanges of secondary structures, another method of creating novel enzymes is the exchange of functional domains. The analogy here is to imagine the functional domains as building blocks that can be exchanged to construct an enzyme that will catalyse a particular reaction. For example, an enzyme whose active site lies at the interface of two domains, with one containing catalytic residues and the other

maintaining specificity, could be amenable to such domain exchanges to create novel enzymes.

As a step in this direction, a functional hybrid glycinamide-ribonucleotide (GAR) transformylase was constructed by fusing a ribonucleotide-binding domain and a cofactor-hydrolase domain as follows[19]. Both GAR transformylase (PurN) and N^{10}-formyltetrahydrofolate hydrolase (PurU) from *E. coli* utilize the cofactor N^{10}-formyltetrahydrofolate. Crystallographic and biochemical information was used to dissect PurN into 2 distinct domains: a ribonucleotide-binding domain and a cofactor-binding domain that appeared to contain residues important in the catalytic mechanism of formyl-group transfer. An equivalent cofactor–catalytic domain in PurU was predicted using sequence homology and secondary-structure-prediction alignments. Several PurN–PurU hybrids were constructed between the PurN ribonucleotide-binding domain and the PurU cofactor–catalytic domain, and these were tested for transformylase activity using a genetic selection: the ability to complement auxotrophic *E. coli* deficient in GAR-transformylase activity. A PurN–PurU hybrid enzyme capable of complementing this mutant was characterized further *in vitro* and found to be capable of the GAR-transformylase reaction, although it favoured hydrolysis of the N^{10}-formyltetrahydrofolate cofactor by 40:1, which was attributed to inappropriate packing of the two domains. This PurN–PurU hybrid also suffered from insolubility problems; similar problems have also been observed in other domain-swapped hybrid enzymes[26]. Difficulties in obtaining correctly folded proteins may be one of the reasons for the low levels of activity observed with other hybrid enzymes.

The creation of restriction enzymes with novel specificities through the fusion of a specific DNA-binding domain with the catalytic machinery of a restriction enzyme is another important application of hybrid enzymes. Type-IIs restriction endonucleases cleave DNA outside their recognition sequence and have been shown to consist of two distinct domains, a recognition domain and a nonspecific DNA-cleavage domain. This class of endonuclease is therefore an attractive candidate for the creation of hybrids, and such a hybrid enzyme has been created by fusing the cleavage domain of the type-IIs restriction endonuclease Fok I with the DNA-binding motif from the *Ubx* homeodomain of *Drosophila* (which contains a helix–turn–helix motif[27]) and with consensus zinc-finger proteins[28]. Although these Fok-I hybrids were able specifically to recognize DNA target sequences and cleave DNA, there were multiple points of cleavage, presumably resulting from nonoptimal positioning of the cleavage domain and differences in the mode of binding of the zinc fingers. The use of zinc-finger proteins as the DNA-binding element is attractive because it should, in theory, be possible to design a zinc finger capable of recognizing any of the 64 triplet codons[29]. It should thus be possible, by linking together a series of engineered zinc fingers, to create a restriction enzyme specific for any DNA sequence.

Enzymes that synthesize secondary metabolites, such as small peptides[30], polyketides[31] and terpenes[32], have also been amenable to hybrid-enzyme manipulation, owing to their modular nature. Nonribosomal synthesis of peptide secondary metabolites is catalysed by multifunctional enzymes whose distinct domains are responsible for specific amino acid activation and modification, and peptide-bond formation; the arrangement of these domains determines the number and order of amino acids in the peptide. One such multifunctional enzyme is the SrfA complex in *Bacillus subtilis*, which generates peptides for the synthesis of the lipopeptide antibiotic surfactin[30]. Using a building-block approach (i.e. that it is possible to synthesize any defined peptide by the proper number and arrangement of activating domains), Phe-, Orn- and Leu-activating domains from *Bacillus brevis* and Cys- and Val- domains from *Penicillium chrysogenum* were individually exchanged for a Leu-activating domain in *srfA*. These hybrid genes encoded peptide synthetases with the desired amino acid specificities and produced peptides with the corresponding amino acid substitutions.

Polyketides are a group of secondary metabolites constructed through the repeated condensation of acetyl and malonyl units to yield such biologically important products as antibiotics, anticancer agents and immunosuppressants. The synthesis of these compounds is directed by multifunctional polyketide-synthase enzymes that not only catalyse repeated acyl-thioester condensations but also introduce variability into their products through the use of different monomers and by varying the extent and degree of β-carbonyl reduction after each condensation. As the polyketide-synthase enzymes have a modular structure, one way of generating new compounds of medical significance would be to 'mix and match' the catalytic units or modules in an effort to direct polyketide synthesis. Such experiments, involving the exchange of catalytic units of type-I polyketide synthases, have recently been described. An engineered form of 6-deoxyerythronolide-B synthase (DEBS) from *Saccharopolyspora erythraea* had its ketoreductase domain replaced with either the ketoreductase and dehydratase domains or the ketoreductase, enoyl-reductase and dehydratase domains of the rapamycin polyketide synthase[33,34]. Expression of the two hybrids produced the predicted products, a decarboxylated tetraketide and an eight-membered-ring tetraketide lactone, respectively. Furthermore, Oliynyk *et al.* [also working with an engineered form of DEBS that was fused with a thioesterase (TE) module to give the bimodular polyketide synthase DEBS1+TE], were able to substitute the acyltransferase domain with that from RAPS1, the first multienzyme component of the rapamycin-producing polyketide synthase[35]. This effectively switched the specificity of DEBS1+TE from methylmalonyl-CoA to malonyl-CoA, with the production of a triketide lactone that specifically lacks a methyl group. These experiments illustrate the feasibility of the *in vivo* generation of specific secondary metabolites by domain swapping.

Introduction of catalytic residues into a binding protein

As yet, there has been no report of such a conversion in the literature. Theoretical examples of this would be the introduction of residues into a glucose-binding protein that conferred hexokinase-like properties upon it or the introduction of catalytic residues into an antibody, akin to the creation of catalytic antibodies. For the latter approach, one would first obtain an antibody that

binds the substrate; then, by examining the binding site and comparing it with existing enzymes that catalyse similar reactions, make point mutations or exchange secondary structures in order to create a hybrid capable of catalysis.

Introduction of residues for both catalysis and binding

The pinnacle of the retrofitting approach is to take a suitable scaffold, either a protein from which the active site has been removed or a small-protein scaffold (e.g. that offered by the scorpion toxins[36]), and introduce an active site that will give both the substrate specificity and the catalytic properties desired. The rationale behind the design of active sites in new structural contexts is as follows: it has been noted that enzymes that catalyse similar reactions have similar active-site structures[37]; specifically, the key catalytic residues in a given family of enzymes are known to possess a specific geometric arrangement, as revealed by the analysis of structural information for the serine proteases and esterases[38]; thus, by delineating the geometric arrangement of residues for a given active site, it should be possible to engineer an active site into a given protein fold.

Such an approach, described by Hellinga and colleagues, utilizes the protein-design algorithm DEZYMER[39]. This is used to search through protein structures to identify regions that are appropriately placed to satisfy the desired active-site geometry. Additional changes may also be required to ensure steric complementarity of the active site with the overall protein structure. The DEZYMER algorithm was used to introduce a nonheme-iron active site (analogous to that found in iron-dependent superoxide dismutase) into thioredoxin, a protein that does not naturally contain an iron-binding site[40]. The resulting enzyme was found to possess one high-affinity metal-binding site and shown to be capable of superoxide dismutation at a rate of $10^5\ M^{-1}\ s^{-1}$, compared with the rate of the naturally occurring enzyme of $10^9\ M^{-1}\ s^{-1}$. The same group has also recently introduced a $[Fe_4S_4]$ cuboidal cluster into thioredoxin[41].

Fusion proteins

Fusion proteins are created by end-to-end fusions of whole genes that encode intact functional proteins and thus can also be considered to be hybrid enzymes. Such fusions have been useful in protein expression and purification[42,43], the display of proteins on the surfaces of cells[44] and phage[45], cellular localization[46] (e.g. targeted delivery of toxic activities to disease-causing cells), metabolic engineering[47], and in the study of protein folding[48]. Fusion proteins are used for screening protein–protein interactions in such systems as the yeast two-hybrid system[49] and small-ligand–receptor interactions in the three-hybrid system[50].

Hybrid enzymes for determining structure–function relationships

Hybrid enzymes have often been used to determine the differences between related enzymes, identifying those residues or structures that impart a specific property that one enzyme has but another, homologous, enzyme does not. For example, hybrids between two highly homologous proteinases from *Lactococcus lactis* were used to determine which residues were responsible for their cleavage specificity and rate towards α_{s1}- and β-casein[51]. The hybrids were also used to identify an additional unique domain involved in substrate binding that was absent from related subtilisins. Hybrid enzymes have also been used to investigate the relative merits of structural and sequence alignments between related enzymes[52].

Methods for the creation of hybrid enzymes

When exchanging units of secondary structure or partial (or even whole) domains, it can be difficult or impossible to create seamless joining of the two sequences by routine gene-cloning techniques. Unless restriction-enzyme sites are fortuitously at the correct locations, or can be engineered by silent mutations[53], cloning will result in extra and/or altered amino acids at the junction of the two sequences.

However, techniques have been devised to circumvent this. Splicing by overlap extension using the polymerase chain reaction[54] can be used to create a seamless fusion, with a few caveats[55]. In addition, vectors have been constructed to create precise fusions by creating unidirectional deletions of intervening bases between the two sequences to be fused[56]. Alternatively, homologous recombination can be used to create nonspecific hybrids between highly homologous enzymes[2].

Most examples of hybrid enzymes are a result of specific, defined protein engineering. However, hybrid enzymes can also be created by the stochastic approach of DNA shuffling[57]. DNA shuffling has mainly been used as a method for directed evolution on a single gene by recursive recombination and mutation[58]. A gene is randomly fragmented and reassembled by error-prone PCR. After some selection process, the iterative process is repeated until a protein with the desired property is successfully produced. DNA shuffling can also be used to shuffle homologous genes to create a library of hybrid enzymes. The degree of homology need not be particularly high: murine and human IL-1β genes, with an average length of sequence homology of only four bases, have been successfully shuffled to create a library of hybrid IL-1β genes[57].

Conclusion

Studies of hybrid enzymes provide a broader understanding of how an enzyme's structure relates to its function and what changes can be tolerated within a particular framework. The ability to engineer the properties of proteins will expand the use of enzymes in biotechnology, and hybrid enzymes created by mimicking nature should play an important role in this endeavor.

Hybrid enzymes have been successfully created by exchanging individual amino acids or secondary and tertiary structures to yield proteins with an altered thermostability, substrate specificity or catalytic property. Although there have been many more examples of successful engineering of nonenzymatic properties such as thermostability, Table 1 illustrates that hybrid enzymes with efficient catalytic properties can be produced. However, the inability to convert chymotrypsin to trypsin[13], and undoubtedly other, unreported protein-engineering failures, illustrates the difficulties

of hybrid-enzyme design. For the most part, hybrid enzymes have, to date, been rationally designed. Recent advances in stochastic approaches, such as DNA shuffling, coupled with emerging selection strategies will probably prove to be important for advances in this field.

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