Review

On the failure of de novo-designed peptides as biocatalysts

(catalyst/artificial enzyme/peptide)

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ABSTRACT While the elegance and efficiency of enzymatic catalysis have long tempted chemists and biochemists with reductionist leanings to try to mimic the functions of natural enzymes in much smaller peptides, such efforts have only rarely produced catalysts with biologically interesting properties. However, the advent of genetic engineering and hybridoma technology and the discovery of catalytic RNA have led to new and very promising alternative means of biocatalyst development. Synthetic chemists have also had some success in creating nonpeptide catalysts with certain enzyme-like characteristics, although their rates and specificities are generally much poorer than those exhibited by the best novel biocatalysts based on natural structures. A comparison of the various approaches from theoretical and practical viewpoints is presented. It is suggested that, given our current level of understanding, the most fruitful methods may incorporate both iterative selection strategies and rationally chosen small perturbations, superimposed on frameworks designed by nature.

A thorough understanding of the chemical and structural bases of biological catalysis would lead to advances in medicine, synthetic chemistry, materials science, agriculture, and other fields. Such a level of insight, when it exists, will likely be signaled by a clear demonstration of the ability to construct, from first principles, a range of catalysts capable of transmuting both biopolymers and small molecules to desired products with high specificity and acceptable efficiency. The specialized proteins known as enzymes are the molecules that usually fill this role in nature, and the most likely means by which we could achieve the objective of catalysis to specification is by attaining an understanding of enzyme structure and function sufficient to allow design and construction of new molecules based on the same principles. This degree of understanding has been elusive, and there is as yet no case of an protein or peptide designed de novo that catalyzes a reaction of biological interest with efficiency and specificity comparable to that of natural enzymes or a novel reaction not carried out by enzymes.

However, competing strategies of developing novel biocatalysts which make use of the frameworks of natural proteins have enjoyed considerable success in catalyzing both transformations analogous to the cognate reactions of natural enzymes and, in some cases, entirely novel reactions. The two major techniques in this category are catalytic antibodies (refs. 1 and 2; for a recent review, see ref. 3) and reengineered natural enzymes (RNEs; for reviews, see refs. 4 and 5). Catalytic antibodies, which were originally reported in 1986, can be characterized as antibodies directed against haptens, which are usually synthetic analogs of the transition states of the chemical reactions to be catalyzed. Reengineering of enzymes has been possible in principle since the advent of genetic engineering, more than two decades ago; a review of Medline entries indicated that studies involving site-directed mutagenesis of enzymes for both reengineering and purely investigatory purposes have produced an average of over 280 papers a year since 1990 in the journals catalogued by Medline. At the opposite conceptual end of the field, chemists interested in catalysis have selected aspects of enzyme behavior capable of being mimicked in smaller systems and have succeeded in producing catalysts with a number of enzyme-like features (6–12). Catalytic RNA or “ribozyme” technology (13, 14) represents yet another promising area in biocatalysis, but it is still possible that the catalytic capabilities of ribozymes, which necessarily have smaller chemical and structural repertoires available than proteins and general synthetic constructs, will prove to be limited to acyl transfer and hydrolysis reactions; these are both areas in which catalytic antibodies and RNEs are already highly proficient. However, hybrid structures involving RNA and peptides, metals, or synthetic compounds will expand the catalytic versatility of nucleic acid structures. There are thus at least four other technologies competing with the designers of catalytic peptides, each with its own level of theoretical advantages, practical feasibility, and demonstrated experimental successes. It may therefore be fruitful to review the major strategies employed to date in advancing the field of biocatalysis and examine the extent to which the hypothetical advantages of each method have been successfully translated into experimental progress. This progress is evaluated both in terms of practical results (i.e., catalysis of novel and/or difficult reactions) and similarity to the behavior of natural enzymes.

Comparative enzymology and protein biochemistry over the past several decades have elucidated several features of natural enzymes that appear to account for most of their catalytic power and versatility (see refs. 15 and 16 for in-depth treatments of this subject). While division of these features into discrete categories depends somewhat on one’s philosophical approach, and several of them are still controversial as to their precise nature and relative importance, it is fair to say that most workers’ views of the essential features of enzymatic catalysis fall into the following five general categories: category 1, entropic effects of enzyme–substrate association and substrate immobilization; category 2, exclusion of solvent from the active site cavity; category 3, transduction of binding energy; category 4, provision of specific chemical groups at fixed locations in the enzyme; and category 5, electronic “tuning” of the active site.

These categories are by no means independent. Category 1 includes both conformational “freezing” and proximity effects. The power of an enzyme to immobilize substrates is also important in bringing reactants into proximity in multisubstrate reactions (17). Category 2 embodies both exclusion of water and other solvent molecules as competitive reagents, and development of a hydrophobic cavity in which the dielectric constant may be vastly different from that observed in solution, thereby enhancing dipole and charge–charge interac-

Abbreviations: RNE, reengineered natural enzyme; PNP, p-nitrophenyl.

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A search of “enzyme” with “site-directed” yielded 1778 references between Jan. 1, 1990 and April 30, 1996. Examination of 49 of these confirmed that all 49 related to the study of enzymes by site-directed mutagenesis. Of course, some journals publishing in this area are not catalogued by Medline.
tions. Category 3 is a complex field; however, both classic and modern experiments have established that the energy of enzyme–substrate association is a critical factor in reaching the transition state (18–22). The full enthalpy attributable to the sum of the favorable interactions in the enzyme–substrate complex is generally not observed in the ΔH of binding, because a portion of the enthalpy of association is reserved as potential energy in the form of conformational strain in the enzyme and/or substrate; this strain may be released as the transition state is approached, or new favorable interactions may be realized. These effects, which collectively reduce the energetic barrier to be overcome in catalysis, are generally known as “transition state stabilization” or “transition state complementarity,” depending on whether one is referring to the energetics or the interactions (15, 16, 23, 24).

Category 4 is the most obvious mechanism by which enzymes promote chemical transformation, but it is closely related to category 1, in that immobilization of the chemical groups in proximity to the substrate is actually an entropic form of rate enhancement, often described as an “effective concentration effect.” Category 5 depends on both the active site environment, which often has a higher dielectric constant than bulk solvent, and the positioning of polar side chains or dipoles composed of multiple residues in the protein. The concept of “orbital steering” (25) holds that the orientations of the molecular orbitals of the chemically active groups of the enzyme and substrate are both fixed in position and electronically tuned by the active site; however, the observation that precise orientation of the prereaction bonds is probably unnecessary (26, 27) is supported by recent experiments indicating that the roles of active site residues may be taken on by substrate moieties (28–32) or even by freely diffusing small molecules in solution (33), which are unlikely to be held in the precise positions occupied by the original groups. This evidence tends to shift the emphasis back toward the entropic effects and the more diffuse polar and hydrophobic influences of the binding pocket, as well as such concepts as “induced fit” (34, 35) and other transduction phenomena.

Of these features of enzymes and enzyme–substrate complexes that are conducive to catalysis, none is fully available to peptides without fixed conformations. Category 1 is virtually ruled out, since stable, many-point contacts between a peptide and another small molecule, neither of which has a fixed three-dimensional structure, are rare. Category 2 is also highly improbable in the absence of a stable binding cavity. Category 3 is not manifestly impossible, but since mechanisms by which the free energy of association is transduced in natural enzymes are poorly understood, it would be astonishing if this effect could be designed and used efficiently in a de novo-designed molecule until further major advances are made in structure prediction and protein dynamics. Category 4 is the feature most readily available to the biocatalyst designer, and the most successful attempts in this field have made free use of contemporary knowledge of both chemical catalysis and the roles of active site residues in enzymes (6–12, 36); however, as mentioned above, our ability to apply this knowledge effectively is severely limited by our failure, for the most part, to deliver appropriate three-dimensional scaffolds for proper placement of the tools of catalysis. Smaller, disordered peptides may contain all the functional groups of enzyme active sites, but even if the peptide is capable of assuming a catalytic conformation, it is conceivable, given the enormous magnitude of shape space accessible to a peptide of even 30 amino acid residues, that the age of the planet would elapse before the desired conformation were to be assumed (37). Finally, while limited provision of polar groups and dipoles as in category 5 may be possible in a peptide, the fixed alignment and hydrophobic cavity, which aim and tune these electronic effects, are likely to be inadequate or absent. In short, all the major features of enzymatic catalysis are likely to be deeply flawed or entirely missing in peptides without fixed conformations.

Against this background, two recent claims of significant advances in the de novo design and construction of biocatalysts surprised the enzyme community and gained some attention in the popular press. The first, which appeared in 1990 (38), reported the synthesis of a complex, branched peptide of 73 amino acid residues (CHZ-1), which mimicked the catalytic activity and specificity of chymotrypsin against three ester substrates, albeit at 0.1–1% of the rate of the native enzyme. Molecular modeling software was used to design a structure that incorporated both the canonical catalytic triad and the oxyanion hole of chymotrypsin. The software model indicated that the complete peptide was able to fold so as to regenerate the chymotrypsin active site with very small positional errors. The alpha-helical dipole that is believed to stabilize the transition state in the natural enzyme was also included in the design. This appeared to be an impressive achievement, since the structure of the peptide apart from the putative active site bore very little resemblance to chymotrypsin. It appeared that the recalcitrant problem of designing a molecule capable of generating an active site which is fixed in space had at least been solved, at least in this case. The designers had taken specific cognizance of categories 4 and 5 above in providing both the active site chemical groups and the “oxyanion hole,” which is thought to provide important stabilization of the transition state in all known serine proteases (39). However, in spite of the molecular modeling computations that had contributed to the design, there was some doubt as to whether a peptide of the size and nature of CHZ-1 was capable of attaining a stable three-dimensional structure. Other de novo–designed peptides of similar nature had been observed to engage in some form of “folding,” but their solution structures appeared to be malleable, a situation similar to the “molten globule” state advanced to explain certain characteristics of folding intermediates in natural proteins (40–42). Moreover, the repeating structure of the “internal” residues of CHZ-1 (those that were intended to form a hydrophobic core between the four helices) allowed the possibility of more than one stable, folded structure. This multiplicity of possible low-energy states has been held to result in a failure to assume a single three-dimensional form in the absence of strongly “fixing” interactions (43, 44).

In 1993, there was a further report of the development of novel biocatalysts, which was even more surprising in many ways. The latter workers claimed to have synthesized two cyclic peptides of 29 amino acid residues that almost precisely duplicated the catalytic capabilities of trypsin and chymotrypsin (45). This in itself was remarkable, but an examination of the peptide sequences showed that they had few stable structural features of any kind other than the expected catalytic triads and some elements of the binding sites, which are adjacent to the active sites of the natural enzymes. The workers had incorporated category 4 in the form of the three residues of the classical serine-protease catalytic triad but had made almost no attempt to include other aspects of enzyme structure. They pointed to the cyclic nature of the peptides as a means of fixing the active site residues in space, but it remained unclear how such a large cyclic structure could maintain conformational stability, and, even if it were capable of doing so, how the putative fixed conformation would relate to the geometry of the natural catalytic triad. The environment of the peptide would be in essence the solvent environment, since there was no provision for exclusion of water or a hydrophobic pocket. As stable, many-point interactions between the substrate and the catalytic peptides appeared to be impossible in the absence of a binding cavity; entropic freezing and transition state complementarity were therefore not to be expected. No dipoles or other electronically active groups were explicitly included, apart from the triad. The workers had addressed
almost none of the well-established requirements for enzyme catalysis yet claimed activity nearly equivalent to that of natural enzymes. If the work was correct, most of mechanistic enzymology was wrong.

Because of the unexpected nature of the latter discovery, many workers in enzymology were anxious to replicate these findings. Shortly after the 1993 paper, two separate groups reported synthesis and testing of the 29-residue peptides (46, 47). Both groups, consisting of highly proficient enzymologists and chemists, failed utterly to observe any catalysis of any hydrolytic reaction by ChPepz and TrPepz. Both sets of workers demonstrated that the peptides were incapable of maintaining stable conformations in solution. Finally, Corey and Phillips (46) pointed out a serious methodological flaw in the kinetics experiments of the original workers: they had used concentrations of the natural enzymes sufficient to exhaust the substrate in the reaction vessel within seconds; the measured rate for the natural enzymes was therefore too low by several orders of magnitude. This error was held to cast doubt on their conclusions about their own synthetic peptides as well. Elucidation of such experimental flaws is very useful to other scientists in the biocatalyst field for two reasons: (i) scientists are able to take cognizance of the flaws in designing future experiments, and (ii) the clarification helps to remove doubt as to whether the failure to reproduce the original results represents errors by the later workers or faulty experimental design or interpretation by those who originally reported the discovery, thereby informing the field at large of the probable fruitfulness of the approach. Finally, to add theoretical grounding to the experimental results in the case of ChPepz and TrPepz, Matthews et al. (48) ably reviewed the state of knowledge of mechanistic enzymology and presented cogent theoretical reasons why small peptides are unlikely to approach natural enzymes in catalytic power; this review was published simultaneously with the refutations written by Corey and Phillips (46) and Wells et al. (47).

We now report our inability to replicate major portions of the work that led to the 1990 report of the catalytic activities of CHZ-1. We have also identified methodological and interpretive errors, similar to the kinetics error identified in the TrPepz work, which cast serious doubt on the accuracy of the claims and conclusions of the original workers in the case of CHZ-1. A report of these results, which constitutes a retraction of sorts, has been published (49), but this article was weakened by both errors of fact and a number of important omissions (through an oversight, the first three authors were not made aware of this manuscript, nor did they approve the venue of publication). In contrast to the original assertions, we have found that: (i) CHZ-1 does not catalyze detectable hydrolysis of benzoyl tyrosine ethyl ester, acetyl tyrosine ethyl ester, or any other substrate tested other than highly labile p-nitrophenyl (PNP) esters; (ii) competitive inhibition is not observed; (iii) thermal denaturation is not observed, even when the peptide is boiled for 3 days; (iv) the catalytic activity of CHZ-1 against the PNP esters increases monotonically with pH with a B value indistinguishable from 1.0, in contrast to the earlier report of a bell-shaped pH-activity profile characteristic of enzymes; and (v) the hydrolytic activity of CHZ-1 against the PNP substrates is considerably lower than previously reported. The major source of these errors appears to have been the omission of important controls in the kinetics experiments. In one case, a PNP substrate was used at a concentration well in excess of its solubility limit; this led to spurious signals due to turbidity, which would have been recognized if adequate control procedures had been performed. Another example is provided by the benzoyl tyrosine ethyl ester experiments. This substrate was dissolved in isopropanol. Addition of this solution to the reaction vessel caused a rise in A256, which was reported as the catalytic rate. We readily demonstrated by means of a no-substrate control that the reported "rate" was independent of the presence of substrate; moreover, experiments in which isopropanol concentrations were equalized between the aliquots to be mixed yielded observed rates of zero. A similar situation was found with the acetyl tyrosine ethyl ester substrate. The rate at which the acid production of the hydrolysis reaction is measured by monitoring changes in pH, was the basis of the remarkably accurate kinetic constants reported in the original paper. However, a review of the methods used raised serious questions about (i) the adequacy of the provisions for exclusion of atmospheric CO2, which has been observed to yield spurious signals in this assay (50, 51); (ii) the methods of accounting for the buffering capacity of the added species in performing the Δ(pH) calculations; and (iii) the adequacy of control procedures performed in the absence of catalyst. We were unable to replicate the original result, and we could not rule out the possibility that acidic or buffering species present in the substrate preparation could have affected the observed pH changes, which in any case were very small. Finally, the original acetyl tyrosine ethyl ester experiments were confirmed by HPLC analysis of the products of hydrolysis. We performed experiments under conditions which were as nearly as possible identical to the original. We observed a much higher background rate of hydrolysis than did the original worker; in fact, our background rate, without catalyst present, was sufficient to account for all of the data reported as evidence of CHZ-1-catalyzed hydrolysis of acetyl tyrosine ethyl ester. We observed no catalysis of this reaction by CHZ-1.

The original report made much of the putative active site of CHZ-1, which had been designed to assume the precise spatial geometry of the well-known catalytic triad common to chymotrypsin and all other known serine proteases. However, when closely similar molecules with both the histidine and the serine of the triad individually replaced or removed were synthesized (histidine was removed, and in a later synthesis, serine was replaced with aminobutyric acid), we found that the catalytic activity against the PNP esters was virtually unaffected. Since these residues have both been shown to be essential for catalytic activity by serine proteases (52, 53), we believe that the active site of CHZ-1, if there is one, is not related to the catalytic triad as designed.

We conclude that the errors in procedure and interpretation in the earlier work have invalidated the claim to enzyme-like catalytic activity by CHZ-1. The peptide does catalyze hydrolysis of certain esters, and is interesting in other ways (for example, even mild digestion of the molecule by trypsin abolishes catalytic activity, indicating that the activity is related to some aspects of the structure, and not merely to the chemical composition). However, the activity meets no critical test of chymotrypsin-like character, such as competitive inhibition by known inhibitors of chymotrypsin, substrate saturation, denaturation by heat, a bell-shaped pH profile, or pronounced specificity for chymotrypsin substrates. We believe therefore that the observed catalytic activity represents a better model for investigation of chemical than of enzymatic catalysis; on such a basis, CHZ-1 may warrant further study. At least two lessons may be drawn from these disappointments. One is specific to the field of kinetics investigations, while the other is a strategic conclusion about the future of attempts to create artificial biocatalysts. First, it must be recognized that the study of enzyme kinetics is quite different from many other fields within the life sciences. In synthetic chemistry, for example, mistakes are discovered by examination of the products; HPLC, TLC, IR, and mass spectrometry generally reveal all. Most experiments in molecular biology are inherently internally controlled. In that DNA sequences may be analyzed and compared with extensive data bases to ensure that what has been cloned or observed is consistent with other known characteristics of the system under study. Kinetics, by contrast, is a quantitative field; an experimental result
may be fundamentally incorrect even though the data obtained are completely self-consistent. Frequentely there is nothing within the results to alert the worker to the error, and mistakes may not be corrected until years after publication. There is therefore good reason to include many independent control procedures to eliminate all possible explanations of the observations other than the hypothesis under test. A general principle has been applied to scientific investigations of so-called “paranormal” phenomena: “extraordinary claims require extraordinary proof.” The same might be said of the field of biocatalytic peptides. The onus is now upon the inventors to supply the proof before making the claims.

The other lesson relates to the proper directions to follow in future attempts to develop novel biocatalysts. No promising field should be excluded from attention, and this certainly includes de novo design. Instead, the issue is the proper apportionment of the resources available to the field of biocatalyst research. With this in mind, it is possible (at a considerable risk of hubris) to review accomplishments to date and extrapolate from these to estimate the likelihood of near-term and medium-term successes proceeding from the various methods. Table 1 displays the advantages, drawbacks, and proven achievements of the five primary techniques that have been used in the production of novel biocatalysts.

Several generalizations may be drawn. Catalytic antibodies and RNEs are highly competitive. So far the advantage lies with RNEs in cases where the reaction is similar to a known enzymatic reaction, and with catalytic antibodies in other cases. Since the category “similar to a known enzymatic reaction” encompasses a broad array of transformations that are of great importance in the life sciences and other fields, RNE technology has many decades of productive progress ahead of it. However, the generality of applications of catalytic antibodies is currently increasing at a faster pace. They are gradually winning the field from RNEs as reagents for organic synthesis. RNEs are still faster catalysts, but catalytic antibodies are catching up, partly through the use of site-directed mutagenesis to introduce improvements to the selected species. Finally, both methods are enjoying the early phases of the advent of biological selections for improved catalytic species (82–84, 90–92). These techniques layer a second, biological selection over the original selective means of producing the catalyst (immune development in the cast of catalytic antibodies, natural selection for enzymes) and thus may be expected to yield very efficient and refined catalytic entities when the selection methodology is perfected and broadly applied. Catalytic RNA is also a very good method for development of specific catalysts within its limited substrate range. Clever applications of specific RNA hydrolysis are currently being applied to medical problems (93–95). By contrast, the de novo techniques have yielded few breakthroughs of practical value. Of the two, de novo peptide design, in spite of the inherent advantages of solid-phase synthesis methodology and the availability of microbial expression techniques, has yielded very little in the way of results of practical or theoretical importance. Although there has been at least one case of catalysis which was based on rational design principles (60), most of the reported examples of catalysis by small peptides have exhibited small rates, and the effects have appeared serendipitously (96–98). On the other hand, chemists working on entirely synthetic constructs have made important progress in simulating various characteristics of enzyme active sites and demonstrating the catalysis of both novel transformations and reactions with stereospecificity. The difference appears to lie in the fact that peptides of the sizes amenable to solid-phase synthesis and conformational analysis do not generally have fixed structures (99), whereas synthetic organic molecules, with a larger range of small units available as input to the design, can be formed with as many bridging and cycling features as desired, providing they are chemically feasible. The most notable successes on the synthetic front have taken full advantage of this capability by creating rigid, cage-like structures, which evidently aid in fixing the positions of the reactive groups on both catalyst and substrate.

As a paradigm of the most recent developments in novel biocatalysts, we offer the example of ADEPT, or antibody-directed enzyme prodrug therapy (100). The concept involves the linkage of a tumor-directed antibody with a (usually hydrolytic) enzyme. A prodrug is introduced into general circulation, consisting of a relatively harmless molecule, which can be activated by enzymatic cleavage to become highly toxic. Injection of the antibody–enzyme complex now completes the strategy. The antibody directs the complex to the cancer site, whereupon the enzyme catalyzes hydrolysis of the prodrug to a poison, ideally one which acts only locally and is degraded before it can be carried to normal tissues. One inherent problem with the idea is that the hydrolytic reaction must be one that is not carried out by any of the enzymes which the prodrug is likely to encounter on its way to the tumor. This generally necessitates the use of an enzyme with no human counterpart, but such a foreign enzyme may cause allergic reactions and other problems. Workers in the catalytic antibody and RNE fields have responded to this need in ways which exemplify the fascinating competitive development of both areas. Catalytic antibodies are already being used to activate produgs (101, 102). Researchers at Glaxo–Wellcome have responded by reengineering a human carboxypeptidase to produce an enzyme that catalyzes hydrolysis of a substrate unknown to biology (ref. 103; R. M. Laethem, personal communication). The prodrug is an analog of this unnatural substrate; its labile bond is cleaved only by the reengineered enzyme, which evidently hydrolyzes no other molecule in the body at significant rates. It will be interesting to see how the catalytic antibody field, as well as other workers in biocatalysis, respond to this development.

Although it does not fall precisely within the category of biocatalysts, another evolving technology deserves mention because of the promise it holds for eventual advances in design and construction of artificial enzymes. Not all de novo protein designers have focused on catalysts. Many have concentrated on structural goals, and in doing so have produced molecules of original design with many characteristics of natural proteins (104–106). Still other workers have attempted to obtain minimized proteins with folded character (107). Having observed the three-dimensional structures of their creations, the designers can proceed to incorporate functions based on the observations, especially liganding capabilities (108–113; see also ref. 104 and references therein). In achieving a balance between information flow from the folding of natural and artificial proteins and the application of rational design principles, the artificial protein workers are proceeding in a manner similar to catalytic antibody researchers, who are reengineering the selected natural antibodies along rational lines (58, 59). Such strategies, incorporating iterative cycles of design or selection followed by information-gathering phases and rationally based perturbations, are intellectually appealing and are evidently well-suited to our current level of insight, since they have yielded considerable experimental success. Individual and intelligently directed applications of our rudimentary comprehension of folding behavior and catalysis, when overlaid on scaffolds designed or selected by natural processes, are yielding increasingly rich theoretical and practical rewards. Conversely, the farther we step away from the information available from the study of natural proteins, the greater is the risk of efforts wasted in attempts to duplicate, with coarse tools and inchoate understanding, the intricate processes of billions of years and uncountable selection events. Like molecular biologists, geneticists, and immunologists, enzymologists too must learn to induce nature to perform as
Table 1. Comparison of methods of developing novel biocatalysts

<table>
<thead>
<tr>
<th>Enzyme-like features</th>
<th>Catalytic antibodies</th>
<th>Reengineered enzymes</th>
<th>Peptides</th>
<th>Other</th>
<th>Catalytic RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entropic effects</td>
<td>Full effect possible</td>
<td>Full effect possible</td>
<td>Severely limited</td>
<td>Possible (6–12)</td>
<td>Full effect possible</td>
</tr>
<tr>
<td>Solvent exclusion</td>
<td>Full effect possible in many cases (54)</td>
<td>Full effect possible</td>
<td>Severely limited</td>
<td>Difficult, but possible (7, 8, 11)</td>
<td>Unclear, possibly unnecessary</td>
</tr>
<tr>
<td>Binding energy transduction</td>
<td>Transition-state complementarity readily achieved, other effects sometimes present (55)</td>
<td>Full effect possible</td>
<td>Virtually impossible</td>
<td>Very limited, but some success reported (7, 8, 10–12)</td>
<td>Important effects possible (56, 57)</td>
</tr>
<tr>
<td>Specific chemical groups</td>
<td>Sometimes present, may be introduced via SDM (58, 59)</td>
<td>Full effect possible, alignment after SDM may be imperfect for catalysis</td>
<td>May be provided (60), but alignment is problematic</td>
<td>Present in natural catalytic RNAs; few successes to date in reengineering active site</td>
<td></td>
</tr>
<tr>
<td>Electronic tuning</td>
<td>No theoretical limitations, varies in practice (61)</td>
<td>Important effects possible with clever engineering (62)</td>
<td>Very limited</td>
<td>Possible (8, 11, 12, 36)</td>
<td>Important effects possible, especially via active-site metal ions (57)</td>
</tr>
<tr>
<td>Experimental success</td>
<td>Entirely novel reactions*</td>
<td>Several successes reported (63–65)</td>
<td>No reported successes</td>
<td>Few reported successes (10, 11)</td>
<td>No reported successes</td>
</tr>
<tr>
<td>Disfavored reactions</td>
<td>Several successes reported (66–68, 114)</td>
<td>Few reported successes†</td>
<td>No reported successes</td>
<td>Virtually no reported successes</td>
<td>No reported successes</td>
</tr>
<tr>
<td>Hydrolysis and formation of peptide and amide bonds</td>
<td>Numerous reported successes, some with activated substrates (69–74)</td>
<td>Not achieved de novo‡</td>
<td>No reported successes§</td>
<td>Very few reported successes (9)</td>
<td>No reported successes</td>
</tr>
<tr>
<td>Enzyme-like substrate specificity, stereospecificity</td>
<td>Excellent (ref. 75 and references therein)</td>
<td>Excellent</td>
<td>Slight (76)</td>
<td>Achievable in some cases (9, 11, 12, 36)</td>
<td>Excellent via base-pairing (56)</td>
</tr>
<tr>
<td>Enzyme-like rates</td>
<td>Not yet achieved</td>
<td>Yes, at times even higher than natural enzymes (77–79)</td>
<td>Not achieved, very improbable</td>
<td>Not achieved, remote at this time</td>
<td>Yes (80)</td>
</tr>
<tr>
<td>Theoretical advantages</td>
<td>Structure selected for catalysis by evolution</td>
<td>Yes, in certain cases (73, 74)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Stable binding clef possible</td>
<td>Yes</td>
<td>Yes</td>
<td>Generally no</td>
<td>Yes, with difficulty (6–11)</td>
<td>Yes, although form is different from those of protein enzymes (57)</td>
</tr>
<tr>
<td>Other theoretical advantages</td>
<td>Readily made complementary to substrate, transition-state analog, or bisubstrate analog</td>
<td>Structure formed by nature specifically for catalysis</td>
<td>Success contributes greatly to theory, unnatural substrates may be incorporated (81)</td>
<td>Sequence specificity is clear advantage when RNA is substrate</td>
<td></td>
</tr>
<tr>
<td>Theoretical limitations</td>
<td>Antibody framework may not be optimal for catalysis</td>
<td>Active site may be restricted to limited range of reactions</td>
<td>Efficient catalysis may be impossible without fixed conformation</td>
<td>No theoretical limitations</td>
<td>Limited structural vocabulary makes catalytic versatility less likely</td>
</tr>
<tr>
<td>Practical advantages</td>
<td>Genetic selection methods available</td>
<td>Yes</td>
<td>Limited to certain systems as yet (82–84)</td>
<td>No reported successes</td>
<td>Yes (85, 86)</td>
</tr>
<tr>
<td>In vitro selection available</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>Site-directed mutagenesis</td>
<td>Yes</td>
<td>For small changes</td>
<td>Appropriate haplotypes must be known and synthetically accessible</td>
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<tr>
<td>Predication of tertiary structure possible</td>
<td>Yes</td>
<td>For small changes</td>
<td>Screening of hyperbodies tedious difficulty</td>
<td></td>
<td></td>
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<tr>
<td>Production of tertiary structure possible</td>
<td>Yes</td>
<td>For small changes</td>
<td>Biological selection methods generally unavailable at present</td>
<td></td>
<td></td>
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<tr>
<td>Means of production</td>
<td>Yes</td>
<td>For small changes</td>
<td>Selection methods generally unavailable at present</td>
<td></td>
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<tr>
<td>Practical difficulties</td>
<td>Yes</td>
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<td>In vivo production straightforward</td>
<td>Yes</td>
<td>For small changes</td>
<td>Selection methods generally unavailable at present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Many RNAs are unstable</td>
<td>No</td>
<td>With limitations depending on complexity</td>
<td>Selection methods generally unavailable at present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vivo production straightforward</td>
<td>No</td>
<td>With limitations depending on complexity</td>
<td>Selection methods generally unavailable at present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Design and prediction of structure extremely difficult</td>
<td>Yes</td>
<td>With limitations depending on complexity</td>
<td>Selection methods generally unavailable at present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Design and prediction of structure extremely difficult</td>
<td>No</td>
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<td>Selection methods generally unavailable at present</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Although enzyme-like catalysis of reactions that are not closely similar to those carried out by known enzymes, have been accomplished in altering substrate specificities and other kinetic parameters, the authors are not aware of any report of the introduction of organophosphate activity into an enzyme.

**Assuming the claims of Atassi and Mandhouri (45) are incorrect, great a proportion as possible of the hard tasks of structural and functional design.
