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Toward Understanding and Tailoring the Specificity of Synthetically Useful Enzymes

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Introduction

The biocatalysis field continues to develop in exciting ways, mainly driven by the synthetic opportunities provided by the exquisite structural specificities and regio- and stereospecificities of enzyme-catalyzed reactions. Many reports of biocatalytic transformations in organic synthesis had been recorded by the late nineteenth century,[1] and the field continued to develop in the early twentieth century, with a broad range of biocatalytic transformations being investigated,[1,2] many of which remain useful today. Even enzymatic reactions in organic solvents had been documented by 1906.[1,3] Despite this early momentum, after the early 1900s little was done until the mid-1950s, when a broad range of preparatively viable transformations of steroids using microorganisms was discovered,[4] resulting in a dramatic breakthrough in cortisone synthesis. In addition, Prelog's work on alcohol dehydrogenases during this era was far ahead of its time.[5] However, it was the growing interest of organic chemists in asymmetric synthesis in the late 1960s that seeded the current renaissance in biocatalysis. Interestingly, the term "asymmetric synthesis" was first coined in 1908 in the context of the enzyme-catalyzed addition of HCN to aldehydes to form cyanohydrin enantiomers.[6] The work on esterases and on alcohol dehydrogenases was initiated in the 1970s by a few pioneering groups[7] and then intensified in the 1980s. Since 1980, the field has grown explosively, with enzymatic reactions in organic solvents further extending the already broad synthetic applicability of enzymes.[8] The current spectrum of opportunities is well documented by excellent reviews and books,[9] by searchable electronic databases,[10] and by an *Organic Syntheses*-like series with independently verified experimental procedures.[11]

Despite the widespread use of enzymes in asymmetric synthesis, the factors that determine their structural specificity and stereospecificity toward the types of new and nonnatural substrate structures of most interest as chiral synthon precursors remain poorly understood. The increasingly broad spectrum of such substrate structures that synthetically useful enzymes are being called on to accommodate makes it essential to delineate the enzyme–substrate interactions that regulate and control enzyme specificity. This will facilitate the identification of the enzymes that are best suited for any particular substrate transformation. It will also guide the development of active site models, and of molecular modeling paradigms, capable of reliably predicting whether an enzyme will accept a new structure as a substrate, and of accurately forecasting the stereochemical outcome of catalysis. Knowledge of the factors controlling enzyme specificity is also of medicinal value in designing inhibitors of enzymes involved in diseases. Some illustrative aspects of these goals are addressed in this Account.

Remote Stereocenter Discrimination

In almost all applications of enzymes in organic synthesis, the stereocenter being introduced or selected is adjacent

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Grace DeSantis received B.Sc. and M.Sc degrees from the University of Toronto. Currently, she is in the final year of her Ph.D. studies in Professor Jones's laboratory, working on chemical modification combined with site-directed mutagenesis to tailor the specificity of subtilisin. She will soon begin postdoctoral studies with Professor C.-H. Wong at the Scripps Research Institute.

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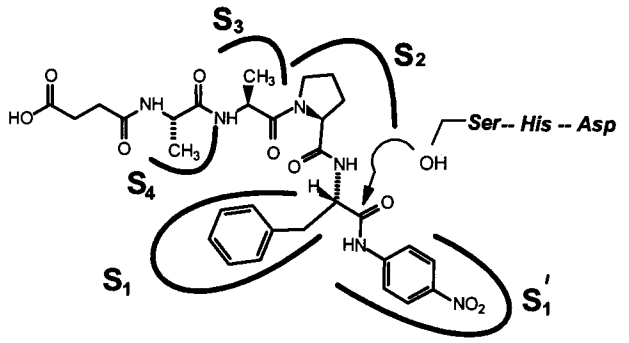


FIGURE 1. Active site of a serine protease, with the succinyl-Ala-Ala-Pro-Phe-pNA substrate bound. The Ser−His−Asp residues of the catalytic triad, and the binding pocket nomenclature[13] are illustrated, with the P_1 residue of the substrate binding in the S_1 pocket while the P_1' leaving group residue binds in the S_1' pocket, etc.

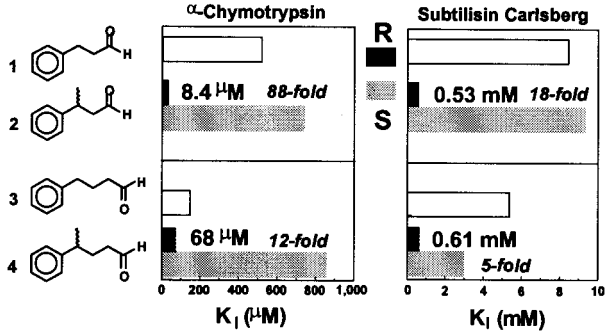


FIGURE 2. Inhibition of subtilisin Carlsberg and α-chymotrypsin by the aldehydes **1**−**4**.

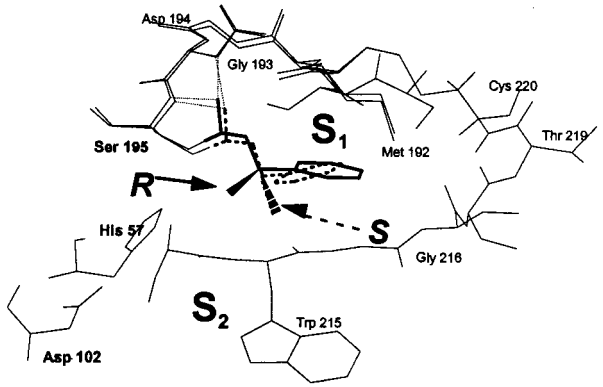


FIGURE 3. Superimposed energy-minimized EI complexes of (*R*)- and (*S*)-3-phenylbutanal, *R*-**2** (—) and *S*-**2** (- - -), in the active site of CT. Both *R*-**2** and *S*-**2** are positioned well into the S_1 pocket and elicit oxyanion hole stabilization via hydrogen bonds between their acetal oxygen and the backbone amide protons of Gly 193 and Ser 195. However, the methyl group at the stereocenter of *R*-**2** (→) is favorably located in the S_2 pocket, while that of *S*-**2** (- - →) points toward the outside of the active site and does not contribute to binding.

to the site of catalysis, and very few examples have been reported where the stereocenter of interest is three or more bonds removed from the carbonyl group of the ester function undergoing hydrolysis. This parallels the situation in nonenzyme-catalyzed asymmetric synthesis, where control of the configurations of stereocenters remote from a chiral auxiliary or catalyst remains a major hurdle. However, since the whole of an enzyme's active site region is chiral, discrimination of any substrate stereocenter is feasible in principle, no matter how remotely such a stereocenter is located from the catalytic site.

These questions regarding the abilities of enzymes to discriminate remote stereocenters have been addressed using subtilisin Carlsberg (SC) and α-chymotrypsin (CT) as representative hydrolases that are commercially available, that have been applied in a wide range of synthetic transformations,[7,9] and whose high-resolution X-ray crystal structures have been determined.[12] SC and CT are serine proteases, each of which has an extended active site binding region composed of several subsites, with the specificity of the S_1 pocket dominating as shown in Figure 1[13] and whose in vivo function is as an endopeptidase which cleaves amide bonds on the carbonyl side of hydrophobic residues.

Evaluating the binding affinities of transition-state analogue competitive inhibitors such as aldehydes represents a convenient strategy for systematic probing of serine protease specificity. Therefore, to evaluate the remote stereocenter stereoselectivity potential of the S_1 pockets of SC and CT, the enantiomeric aldehydes *R*- and *S*-**2**, **4** with remote stereocenters positioned β and γ, respectively, to the carbonyl group, together with their achiral parent structures **1**, **3** as reference compounds were used (Figure 2).[14]

The strongest inhibition (lowest K_I) observed was of CT by (*R*)-3-phenylbutanal (*R*-**2**), whose K_I is 88-fold lower than that of its *S* enantiomer. In contrast, binding of *R*-**2** to SC is only 18-fold lower than that of its *S* enantiomer, demonstrating the inherently lower stereochemically discriminating nature of the S_1 pocket of SC. Nevertheless, both enzymes demonstrate significant remote stereocenter discriminatory capabilities. However, their behavior still parallels that of chemical asymmetric catalysts and auxiliaries in that the degrees of stereocenter discrimination are reduced the further the stereocenter is removed from the site of catalysis, from 88-fold for β to 12-fold for γ for CT, for example.

The basis for the dramatic 88-fold difference in binding of (*R*)- and (*S*)-3-phenylbutanal to CT was explored using molecular modeling (Figure 3) and demonstrated that the main contributor to the better binding of *R*-**2** was the extra hydrophobic binding elicited by locating its methyl group in the S_2 pocket, an interaction that was not available to the enantiomer *S*-**2**. The beneficial binding contribution of the methyl group of *R*-**2** is also reflected by its K_I being 61-fold lower than that of its achiral precursor, 3-phenylpropanal (**1**, Figure 2). Furthermore, the $\Delta\Delta G$ differences between experimental (from K_I's) and calculated (from enzyme−inhibitor (EI) complex energies) for the *R* and *S* pairs of **2** and **4** are quantitatively similar for both CT and SC, confirming the validity of molecular modeling results indicating weaker *R* versus *S* stereocenter discrimination for structures **4** than for **2** and providing an endorsement of the potential of molecular modeling to forecast the degree of stereocenter discrimination.

Reversal of Natural Stereoselectivity. While both CT

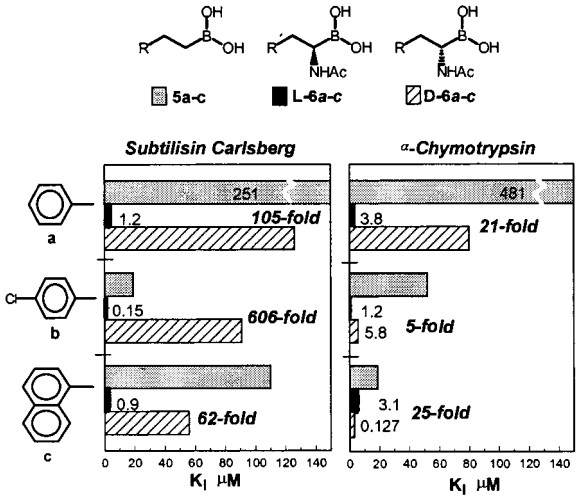


FIGURE 4. Inhibition of subtilisin Carlsberg and α-chymotrypsin by achiral (**5a**−**c**) and chiral (L- and D-**6a**−**c**) boronic acids. An unexpected reversal of the normal specificity was observed for CT, with a 25-fold (lower K_I) preference for D-**6c** than for L-**6c** being observed.

and SC exhibit a dominant stereoselectivity preference for the L-amino acid configuration for the hydrolysis of their natural protein substrates, predicting their stereoselectivities for unnatural substrates is not straightforward. For example, for CT, unexpected reversals of stereoselectivity can even be observed within a homologous series of amino acid ester substrates.[15] In addition, the stereoselectivity of SC and CT can be modified when water is replaced with a nonaqueous solvent[16] or by changing the pressure in supercritical fluids.[17]

In studying this aspect of specificity, we again exploited the good binding characteristics of transition-state analogue inhibitors, in this case with boronic acids.[18] The enantiomeric 1-acetamidoboronic acids L-**6a**−**c** and D-**6a**−**c**, which are competitive transition-state analogue inhibitor mimics of L and D forms of the *N*-acetylamino acids phenylalanine, *p*-chlorophenylalanine, and 1-naphthylalanine, respectively, were prepared and, together with their unsubstituted boronic acid parents **5a**−**c**, were evaluated as inhibitors of SC and CT.[18] All of the boronic acids proved to be powerful competitive inhibitors of both enzymes, as summarized in Figure 4.

For SC, each L enantiomer is a more potent inhibitor than its D counterpart. Furthermore, the *p*-chloro group contributes very positively to inhibitor binding (cf. **6a** vs **6b** and **5a** vs **5b**) due to the interactions of the para-electronegative substituent with the region of positive potential identified at the bottom of the S_1 pocket of SC.[19] For CT, the L enantiomers are also generally more potent inhibitors than are their D counterparts. However, a totally unexpected reversal of the L fidelity of CT was observed for binding of (1*S*)-acetamidonaphthylboronic acid (D-**6c**), which is a 25-fold more potent inhibitor than L-**6c**, and whose phenomenally low K_I of 127 nM represents the strongest binding of any simple boronic acid inhibitor of serine proteases. To account for this puzzling stereoselectivity reversal, we again turned to molecular modeling. Of the aromatic residues of **5a**−**c** and **6a**−**c**, only the naphthyl groups of L- and D-**6c** lack C_v symmetry. This offered a possible basis for rationalizing the stereoselectivity reversal, since rotation of the naphthyl group about the σ-bond could give rise to conformationally distinct EI complexes, as illustrated schematically in Figure 5. Molecular modeling supported this concept, showing the naphthyl group of both L-**6c** and D-**6c** binding preferably to SC in the right-oriented conformation, and to CT in the left orientation.

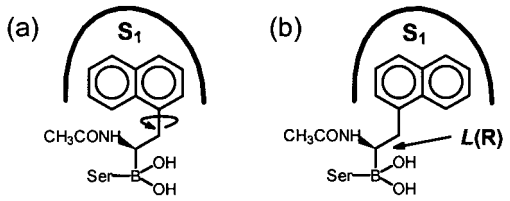


FIGURE 5. Left-oriented (a) and right-oriented (b) naphthyl conformations resulting from rotation about the σ-bond of L-**6c**.

However, formulating appropriate molecular modeling protocols for this study was not straightforward because of the sometimes fickle nature of boronic acid binding to serine proteases. Boronic acids are capable of forming tetrahedral complexes with either the active site serine or histidine residues,[20] and generally, good substrate analogues bind to serine and poor substrate analogues bind to histidine. To address these issues rigorously, we obtained the X-ray structures of the SC and CT complexes with L- and D-**6b**,**c**.[21] These revealed that, as expected, for both enzymes the L enantiomers of the inhibitors formed tetrahedral adducts, with the O_γ atom of the catalytic serine covalently linked to the boron atom. Surprisingly, the D enantiomers differed in the way they interacted with subtilisin and chymotrypsin, and their binding modes were not as predicted by NMR.[20] With subtilisin, both D-**6b** and D-**6c** behaved like their L counterparts, forming covalent Ser 221 O_γ-to-boron bonds.[20] In contrast, and very unexpectedly, with CT the EI complexes with D-**6b** and D-**6c** gave novel tetrahedral adducts in which *two* covalent bonds to boron were observed, one with His 57 $N_{\epsilon 2}$ as forecast[20] and the other with Ser195 O_γ as shown in Figure 6. This situation was quite intriguing since, although binding of a boronic acid inhibitor to give either a serine or a histidine adduct has been reported,[22] no tetrahedral adducts simultaneously linking boron covalently to both serine and histidine have previously been documented. Furthermore, the outstanding binding of D-**6c** to CT is elicited without any oxyanion hole stabilization of the tetrahedral EI complex. Clearly, much remains to be done in identifying the factors controlling binding of inhibitors and substrates to enzymes. However, the potential of molecular modeling in this regard was underscored by the fact that the different naphthyl conformations identified by modeling to account for the stereoselectivity differences between SC and CT binding of the **6c** enantiomers were supported by the X-ray data on the EI complexes of L- and D-**6c** with SC and CT, which confirmed the expected left and right orientations of the naphthyl groups, respectively (Figure 5).

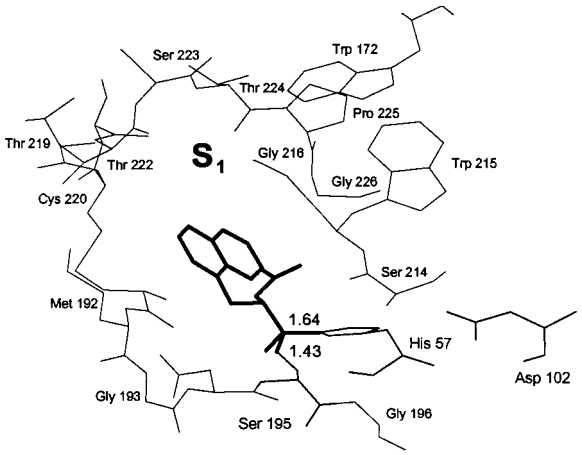


FIGURE 6. D-**6c** bound to CT showing the naphthyl ring bound in the S_1 pocket, the 1.43-Å B-to-O_γ Ser covalent bond, and the 1.64-Å B-to-$N_{\epsilon 2}$ His covalent bond.

Inhibiting the Enzymes of Disease: *β*-Lactamases. While the comparisons of X-ray data with molecular modeling structures described above reveal deficiencies in the modeling protocols, the inhibitor design strategies involved are soundly based and can be reliably used to design inhibitors of enzymes involved in diseases, such as β-lactamases. β-Lactamases are of current interest due to their key role in the development of bacterial resistance to β-lactam antibiotics, and the therapeutic application of β-lactamase inhibitors represents one strategy for overcoming antibiotic resistance. The most effective β-lactamase inhibitors described thus far[23] are themselves β-lactams. With the challenges posed by continuing bacterial resistance, identification of new β-lactamase inhibitors is of considerable clinical and mechanistic interest. Boronic acid inhibitors have already provided useful information in this regard and have been shown by ^{11}B NMR spectroscopy to be reversible transition-state analogue inhibitors that form tetrahedral adducts with the active site serine of β-lactamases.[24]

Employing the X-ray structure of the class A RTEM-1 β-lactamase from *Escherichia coli*, and with the representative cephalosporin substrate−enzyme interactions outlined in Figure 7a as a guide, the boronic acid transition-state analogue inhibitors **7a**,**b** were designed. It was envisaged that the electrophilic boron would interact with the active site Ser 70 to form a tetrahedral intermediate stabilized by the oxyanion hole and that the acetyl side-chain orientation would achieve the desired hydrogen-bonding interactions with Ala 237 and Asn 132. The position of the carboxylate moiety was selected to elicit strong electrostatic interactions with the Arg 244, Ser 235, and Lys 234 side chains. The *N*-acetyl (**7a**) and N-phenylacetyl (**7b**) groups were included in order to evaluate the contribution of the *N*-acyl side chain in the binding of the target inhibitors.

Kinetic evaluations of **7a** and **7b** revealed them to be highly effective, slow binding, competitive inhibitors of the class A RTEM-1 β-lactamase. The 110 nM K_I of **7a**,[25] in which the *N*-acyl side chain is an acetyl group, is reduced by 19-fold when the *N*-acyl function becomes the much more hydrophobic phenylacetyl side chain, as in **7b** with its dramatically lower K_I of 5.9 nM.[26] The carboxylate moiety is critical for nanomolar inhibition since the K_I's of **7c** and **7d** are 96 and 351 μM, respectively.[30] The X-ray structure of the EI complex between (1*R*)-1-acetamido-2-(3-carboxyphenyl)ethylboronic acid (**7a**) and the RTEM-1 β-lactamase[27] shown in Figure 8, reveals that the complexed boron atom is indeed tetrahedral as expected and that the inhibitor interacts with the enzyme's active site essentially as designed (Figure 7).

The rational design approach depicted in Figure 7 is clearly a very effective one, with the affinity of inhibitors **7a** and **7b** comparable to those of the most powerful mechanism-based inhibitors, such as the penem BRL 42715[23] and olivanic acid derivatives,[23] and to those of the clinically used clavulanic acid, sulbactam, and tazobactam.[23] We have also successfully applied similar approaches in the design and synthesis of highly effective conformationally restricted analogue inhibitors of medicinally relevant cysteine proteases.[28]

Tailoring and Probing Stereospecificity and Structural Specificity. Applications of oxidoreductases with broad specificities, such as horse liver alcohol dehydrogenase (HLADH), in asymmetric synthesis have been extensively studied.[29] To complement these broad specificity enzyme data, we therefore decided to examine an oxidoreductase with narrower specificity, with the intent of further delineating the factors determining structural specificity and stereospecificity. The L-lactate dehydrogenase of *Bacillus stearothermophilus* (BSLDH) is an excellent candidate for such studies since it is a very stable, modestly thermophilic, enzyme of known protein sequence and its properties have already been the subject of several studies.[30] BSLDH is an NAD/H coenzyme-dependent, oxidoreductase enzyme whose in vivo function is to catalyze the interconversion of pyruvate (**8**) and L-lactate (L-**9**) as shown in Figure 9a.

BSLDH is one of the most stereospecific enzymes known[31] and thus represents an excellent instrument for probing stereospecificity determinants. Among the methods of exploring the factors controlling stereospecificity, evaluating how effectively an enzyme resists attempts to change this inclination is potentially one of the most informative. In the case of BSLDH, its natural L stereospecificity is a consequence of the orientation of pyruvate (**8**) in the ES complex such that the hydride equivalent from NADH is delivered to the *Re* face of the carbonyl group. This situation is represented schematically in Figure 9a. A key interaction helping to maintain the required pyruvate orientation is that between the substrate's COO^- and Arg 171. Conversely, reduction of pyruvate to D-lactate would require the "hydride" of the NADH coenzyme to be delivered to the *Si* face of pyruvate. Inducing this *Si* face attack via an ES complex in which the orientation of pyruvate is "flipped" was envisaged, as illustrated in Figure 9b. This strategy entails exchanging the natural COO^- binding (of Arg 171) and hydrophobic, CH_3 side-chain binding (of Gln 102 or Ile 240) sites by introductions of 171Tyr or 171Trp, or 102Arg or 240Arg/

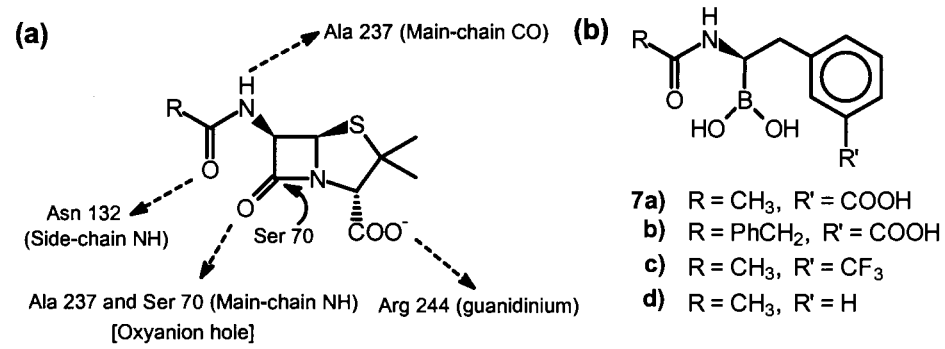


FIGURE 7. (a) Schematic β-lactamase−cephalosporin Michaelis complex and (b) its inhibitor mimics.

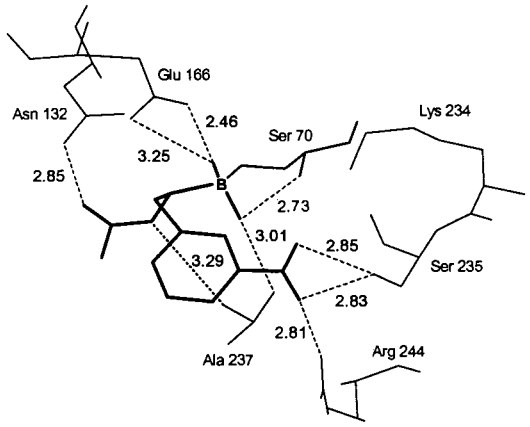


FIGURE 8. X-ray structure[27] of EI complex between **7a** and the RTEM-1 β-lactamase showing the multiple hydrogen-bonding and electrostatic interactions elicited. Distances are shown in angstroms.

Lys, respectively, and therefore several single and double mutants of these kinds were created.

The greatest degree of stereospecificity reversal achieved was a very significant 2.3% of D-lactate formed by the Ile240Lys/Arg171Tyr double mutant. This represents a truly remarkable >500-fold switch in stereospecificity preference, from an experimentally determined D enantiomer formation frequency of <1 in 25 000 for WT-BSLDH to 1 in 43 for the Ile240Lys/Arg171Tyr mutant. Furthermore, LaReau and Anderson[31] have demonstrated that for NAD^+, nonstereospecific hydride transfer to the "wrong" *Si* face of the nicotinamide ring occurs at most in <1 of 10^7 reactions and it is reasonable to assume that the stereochemical integrity of the hydride transfer from NADH to the *Re* face of the carbonyl group of pyruvate is comparable. Based on this precedent, the 2.3% D-lactate production observed with Ile240Lys/Arg171Tyr represents a significant $>2 \pm 10^5$-fold relaxation of the L stereospecificity relative to that of WT-BSLDH. Nonetheless, the existence of a network of secondary "fail-safe" interactions which BSLDH can invoke to maintain the substrate in its natural orientation is evident since even the most successful Ile240Lys/Arg171Tyr double mutant is still >97% L stereoselective.[32] Interestingly, a comparison of the active sites of L- and D-LDH's reveals them to be virtual mirror images of one another, demonstrating that nature has adopted a similar "reversed" substrate binding mode strategy to produce D-lactate.[32,33] We now plan directed evolution of BSLDH toward D stereoselectivity and predict that the active site of the "new" D enzyme will mirror that of natural BSLDH.

An additional serendipitous benefit derived from the above mutations is that the Arg171Tyr and the Arg171Tyr/Gln102Arg mutants displayed dramatically increased thermal stabilities. For example, the Arg171Tyr/Gln102Arg enzyme retains 30% of its activity even after heating for 30 min at 100 °C whereas the WT enzyme is completely inactivated in less than 2 min.[34] The increased thermal stability is of considerable synthetic benefit since, subject to NADH stability, preparative-scale BSLDH-catalyzed reactions at temperatures of up to 100 °C can now be contemplated. The increased thermal stability on replacing the Arg 171 by hydrophobic residues such as Trp is attributed to more favorable hydrophobic subunit contacts.

While BSLDH has a narrow structural specificity, it will accept a broad range of other α-keto acids as substrates, albeit with substantial rate penalties for large or branched *R* groups.[35] However, these penalties can be ameliorated by creating more room for the larger substituents, such as those of **10a**−**d**, by replacing Gln 102 (Figure 10a) by smaller amino acid residues, such as Asn.[35] In addition, by substituting Gln 102 by the acidic amino acid residues Asp or Glu, an active site is created that will accept positively charged side chains, as shown in Figure 10b. When this is done, γ- and ϵ-amino-α-keto acids such as **10e**,**f**, which are very poorly accepted by the WT enzyme, then become excellent substrates for the Gln102Asp/Glu mutants.[36] Conversely, the Gln102Arg mutant favors dicarboxylic acid substrates such as **10g**−**i** (Figure 10c).[35]

A New Approach: Chemical Modification of Mutant Enzymes

While protein engineering has emerged as a practical technique to alter enzyme specificity, it is restricted to natural amino acid replacements, and the biological[37] and chemical modification techniques[38] recently devised to address this limitation are not readily amenable to routine or large-scale applications. In this regard, we have begun to explore the combination of site-directed mutagenesis and chemical modification as a powerful and versatile technique for the creation of new active site environments and for mechanistic studies. This strategy involves the introduction of a cysteine residue at a key active site

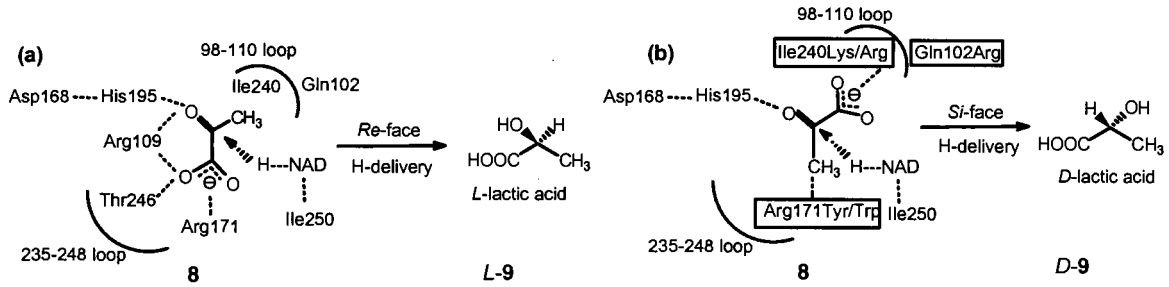


FIGURE 9. (a) Schematic representation of the natural binding mode of pyruvate (**8**) at the active site of BSLDH in orientations leaing to L-lactic acid (L-**9**) due to *Re* face hydride delivery. (b) This illustrates a reversal of the natural binding orientation of pyruvate that might be induced by mutations such as Arg171Trp/Tyr, Gln102Arg that would "flip" the pyruvate binding site and lead to D-lactate by *Si* face hydride delivery.

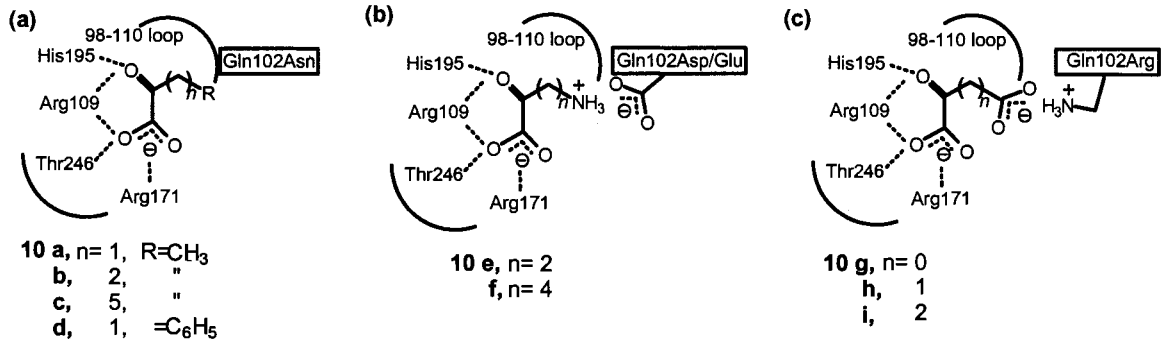
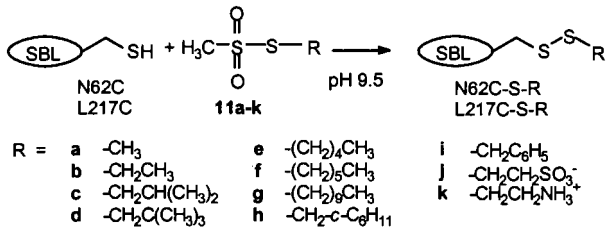


FIGURE 10. Active site BSLDH representations illustrating site-directed mutations that improve steric tolerance of larger groups (a) and improve electrostatic interactions for positively (b) and negatively (c) charged substrate side chains of α-keto acid substrates.

Scheme 1



position via site-directed mutagenesis which is then thioalkylated with an alkyl methanethiosulfonate reagent (**11a**−**f**) to give a chemically modified mutant enzyme (CMM) as illustrated in Scheme 1. Alkyl methanethiosulfonate reagents react specifically and quantitatively with thiols and are routinely used for chemical modification of protein thiols. The modification can be performed under mild reaction conditions, on a large scale, can be reversed by treatment with β-mercaptoethanol, and is independent of the nature of the R group. An unlimited range of structural variations can therefore be introduced into the enzyme.

We employed the alkaline serine protease subtilisin *Bacillus lentus* (SBL) to evaluate this strategy since it is a well-characterized enzyme of synthetic and industrial interest, and whose high-resolution crystal structure has been solved.[39] Also, it has been cloned, overexpressed, and purified and its kinetic behavior well characterized. More importantly, WT-SBL contains no natural cysteine residues and methanethiosulfonate reagents therefore react *only* with the cysteine residue introduced by mutagenesis. Our initial goal was to alter the specificity of

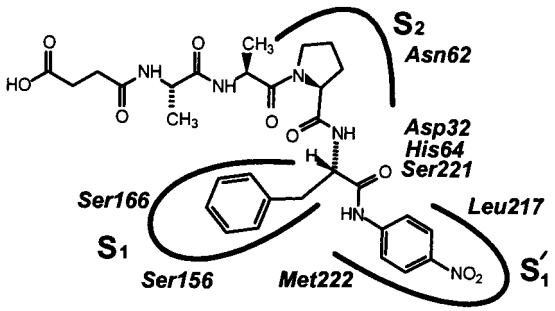


FIGURE 11. Representation of the active site of SBL with the succinyl-Ala-Ala-Pro-Phe-pNA substrate bound. The catalytic triad is composed of Asp 32, His 64, and Ser 221. The residues chosen for mutagenesis and modification are Ser 156 and Ser 166, which are in the S_1 pocket, Asn 64, which is located at the opening of the S_2 pocket, Leu 217, which is located at the opening of the S_1' pocket, and Met 222, which is buried and is adjacent to the oxyanion hole and catalytic Ser 221.

the S_2, S_1, and S_1' pockets of SBL, since binding of substrate groups at these sites is the most frequently exploited in synthesis. Using the X-ray crystal structure of SBL as a guide, the residues identified in Figure 11 were selected for mutagenesis and modification.

This new approach provides a broad range of specificity tailoring opportunities,[40] but in this Account we will focus on only one illustrative example of what can be achieved. All too often, site-directed mutagenesis creates mutant enzymes with lower than WT activities. We therefore set ourselves the goal of creating CMMs that could at least match the activity of the WT parent. With this objective in mind, SBL-N62C and -L217C (Scheme 1) were reacted with the methanethiosulfonates **11a**−**k**.[41,42]

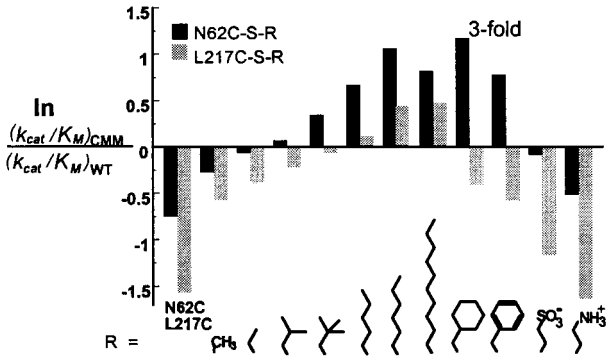


FIGURE 12. Plot of $\ln[(k_{cat}/K_M)_{CMM}/(k_{cat}/K_M)_{WT}]$ for N62C and L217C CMMs. This shows enzymes with higher activity as bars above the *x*-axis and those with lowered activity below.

Despite the breadth of the structural range of the *R* groups introduced at L217C and N62C (Figure 12), virtually all of the chemical modifications resulted in CMMs with higher k_{cat}/K_M's than their cysteine parents, with L217C-S-**k** being the single exception to this pattern. However, for either site, introductions of the charged sulfonatoethyl (R = **j**) and aminoethyl (R = **k**) groups were only marginally beneficial relative to those of any of the hydrophobic moieties (**a**−**i**). It also became evident that the more hydrophobic the introduced modifying group, the higher the k_{cat} values, with the N62C CMMs being more consistently activated than their L217C analogues. It is remarkable that, of 22 CMMs evaluated, almost half of them not only reached the wild-type k_{cat}/K_M level, but in fact exceeded it by factors of up to >3.[41] To our knowledge, the attainment of these levels of augmented WT activity for such a broad range of modified enzymes is unprecedented and has not been matched so far in its breadth by protein engineering methods alone. Preliminary data suggest that the significant activity enhancements achieved by the hydrophobic modifications, particularly of the N62C series, are attributable to alterations in their pH−activity profiles arising from changes induced in the pK_a of the catalytic His 64.[43] Further CMM-based alterations of SBL's structural specificity and stereospecificity are proceeding on a broad front.

Future and Perspective

Virtually all classes of reactions achievable by conventional organic synthesis now have an enzyme counterpart.[7,9,44] However, in the enzyme arena, to date we have been content to exploit only the enzymes that are currently operational in nature. There are undoubtedly "silent" genes that can be turned on to generate new enzyme catalysts by exposing cells to the right substrates. The induction of β-lactamase by bacteria in becoming resistant to β-lactam antibiotics and the toluene dioxygenase induced in *Pseudomonas putida* from chemical dumps, for cis dihydroxylation of benzene derivatives, are just two such examples. Also, plant enzymes represent an underused resource. This is illustrated by the recent discovery of what appears to be the first authentic Diels−Alderase. Previous to this, catalytic antibodies represented the only biocatalytic prospect for this reaction. Furthermore, the synthetic potential of RNA enzymes is beginning to be documented.[44]

Full exploitation of the synthetic potential of enzymes can be anticipated by applying insights into enzyme specificity gained from structure−activity studies, such as those described above. In addition to such rational tailoring approaches, novel biocatalyst creation via directed evolution will undoubtedly expand rapidly. The potentials of unnatural amino acid mutagenesis and CMM technology will also develop. The de novo design of enzymes, while still in its infancy, can be expected to make dramatic progress soon, as can biocatalyst creation by taking an existing "protein scaffold" possessing a desired specificity and grafting an alternate catalytic machinery onto it. Approaches involving DNA shuffling and metabolic pathway engineering, where cell metabolism is manipulated in a desired synthetic direction through genetic and modular engineering coupled with the use of nonnatural enzyme substrates, also promise to grow rapidly.

Despite the already enormous spectrum of biocatalytic opportunities, we look toward the future with a great deal of excitement as competing and complementary technologies are further developed, as enzymes more compatible with organic solvents and higher temperatures become increasingly available, as screening of microorganisms and protein engineering methods become still faster, and as new methods to prepare viable catalytic antibodies, such as reactive immunization and direct selection of catalysis, develop. All of this will be facilitated as X-ray structures become even more routinely available, as better paradigms for predicting the factors controlling and determining enzyme 3D structures are developed, and as molecular modeling methods become more dependable. Furthermore, biocatalysis has emerged as the basis of a powerful "green" chemistry which will be increasingly promoted by environmental concerns for the large-scale synthesis of compounds in medicinal chemistry and for the creation of new, biodegradable, materials.

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