Enzymes in Organic Synthesis. Present and Future


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A utilidade e a praticidade do uso de enzimas como catalisadores quirais em síntese orgânica estão bem documentadas. A sua aplicação na criação de synthons enantiomericamente puros em sínteses assimétricas de produtos naturais de interesse acadêmico e farmacêutico é particularmente importante. Esterases e oxidoredutases são grupos enzimáticos de utilidade comprovada nesta área. Os tipos de oportunidades de preparação de synthon quirais que tais enzimas oferecem são ilustrados, juntamente com modelos de uso simples dos seus sítios ativos.

Entretanto, apesar da generalizada exploração de enzimas para fins sintéticos tanto em aplicações acadêmicas como em industriais, pouco se sabe sobre os fatores que determinam a especificidade da enzima, ao mesmo tempo que se amplia rapidamente o espectro de estruturas de substrato não-naturais com as quais se espera que as enzimas sinteticamente úteis sejam capazes de lidar. Desse modo, está se tornando essencial delinear as interações enzima-substrato que regem a especificidade estrutural e a estereoespecificidade, para assim facilitar a identificação das enzimas mais adequadas para transformar novas estruturas de substratos nos synthons quirais desejados. Informação similar tem utilidade medicinal no desenho de inibidores de enzimas envolvidas em doenças. Algumas estratégias são apresentadas para sensoar especificidades de enzimas em ambas estas categorias.

Não se pode esperar que enzimas naturais sejam capazes de aceitar todas as estruturas de interesse quimio-sintético, nem que sempre as transformem estereoespacialmente nos almejados materiais enantiomericamente puros que se necessita em síntese. Assim, no futuro será necessário o desenvolvimento da capacidade de alterar, de um modo controlado, as especificidades de enzimas. Estes objetivos estão sendo perseguidos através da engenharia de proteínas. Resultados ilustrativos dessas tentativas de efetuar mudanças controladas da especificidade são descritas, usando-se uma desidrogenase L-lactata como representativa das oxidoredutases.

The usefulness and practicality of enzymes as chiral catalysts in organic synthesis are now well documented, with their applications in creating enantiomERICALLY pure synthons for the asymmetric synthesis of important natural products of academic and pharmaceutical interest being particularly important. Enzyme groups of proven value in this regard are the esterases and the oxidoreductases. The types of chiral synthon preparation opportunities that such enzymes offer are illustrated, together with their simple-to-use active site models.

However, despite the widespread exploitation of enzymes for synthetic purposes in both academic and industrial applications, little is known about the factors that determine enzyme specificity, while the spectrum of unnatural substrate structures that synthetically useful enzymes are required to handle continues to broaden rapidly. It is therefore becoming essential to delineate the enzyme-substrate interactions that regulate structural specificity and stereospecificity in order to facilitate the identification of the enzymes best suited to transforming new substrate structures into the desired chiral synthons. Similar information is of medicinal value in designing inhibitors of enzymes involved in diseases. Some illustrative strategies for probing enzyme specificities in both these categories are presented.

Natural enzymes cannot hope to accept all structures of synthetic chemical interest, nor always to transform them stereospecifically into the desired enantiomERICALLY pure materials needed for synthesis. Thus, in the future it will become necessary to develop the capability to alter the
specificities of enzymes in a controlled manner. These goals are being addressed by protein engineering. Illustrative results of such approaches to effect controlled changes of the specificity are described, using an L-lactate dehydrogenase as a representative oxidoreductases.

Keywords: enzymes in organic synthesis, enzyme inhibitors, protein engineering

Introduction

Enzymes are now widely applied as catalysts for a broad range of organic synthesis, particularly for inducing asymmetric transformations1. However, despite the widespread uses of both enzymes and microorganisms in asymmetric synthesis, relatively little is known of the factors that determine their structural specificity and stereospecificity. The increasingly broad spectrum of new and unnatural substrate structures that synthetically useful enzymes are being called on to accommodate, makes it increasingly 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 given chiral synthon preparation. It will also guide the development of active site models capable of reliably predicting whether an enzyme will accept a new structure as a substrate, and of accurately forecasting what the stereochemical outcome of the reaction will be. Knowledge of the factors determining enzyme specificity is also of medicinal value in designing inhibitors of enzymes involved in diseases. Furthermore, an understanding of the factors determining specificity will facilitate the rational tailoring of enzyme specificity by the site-directed mutagenesis techniques of protein engineering. Representative aspects of all of these goals are addressed in this paper.

Two of the classes of enzymes most widely applied synthetically are the hydrolases and the oxidoreductases. This perspective will focus on probing the specificity of representative, synthetically useful, members of both of these groups. The range of transformations accessible by such enzymes are illustrated in Fig. 1 for horse liver alcohol

![Diagram of enzymatic transformations](image_url)

**Figure 1.** Illustrative horse liver alcohol dehydrogenase (HLADH)- and pig liver esterase (PLE)-catalyzed transformations of asymmetric synthetic value.
dehydrogenase (HLADH) and pig liver esterase (PLE). The types of chiral synths generated in these ways have been exploited in the asymmetric syntheses of a broad range of biologically and structural significant targets, as exemplified in Fig. 2.

For any synthetic process, the ability to predict the accessible range of structures, and the stereochemical outcome of the reaction, is of primary importance. Presently, for enzyme-catalyzed reactions, such analyses are performed using active site specifications of the Fig. 3-type. The model shown for PLE is representative, with others for porcine pancreatic lipase and HLADH also having been developed in our laboratories. Because of their simplicity and ease of application, such active site formulations are presently the predictive models of choice of organic chemists using enzymes synthetically, even when X-ray structures are available. However, since such models are empirically derived, they do not provide the basic understanding of enzyme specificity that is essential for selecting the most appropriate enzyme for transforming a given substrate structure into a desired synthon and for identifying the range of substrate structures any synthetically useful enzyme can accept. Accordingly, in order to delineate the factors that control and determine enzyme specificity, we have begun to probe the nature of enzyme-substrate interactions in a systematic manner. Our strategy involves studying synthetically useful, and also medicinally relevant, enzymes for which good X-ray structures are available, and using graphics analyses to select substrate or inhibitor structures that address a particular specificity question most appropriately. Then, kinetic studies are performed on the selected structures, and the experimental data are analyzed with the aid of graphics and molecular modelling. Our ultimate objective is to maximize the synthetic scope of each enzyme, and to optimize the design of inhibitors of the enzyme of disease for use as potential drug candidates. In addition, since no natural enzyme can be expected to handle the range of substrate structures imposed by the chiral synthons demands of asymmetric synthesis, an additional benefit of this approach is that it will identify amino acid residues at an active site that preclude conversion of a synthetically desirable substrate structure. This opens up the possibility of using the site-directed mutagenesis techniques of protein engineering to ameliorate unfavourable amino acid positions, and eventually to

![Diagram](image)

**Figure 2.** Some illustrative chiral target molecules synthesized from enantiomerically pure enzyme-generated precursors.
studies probing and modifying the structural specificities of the S1 sites of serine proteases with the most recent directed towards evaluating electrostatic effects.

The calculated (using the BioSym Delphi programme) electrostatic potential surfaces of SC and CT are very different, thereby raising the possibility that electrostatic differences could be exploited to improve the strength or selectivity of binding to enzymes for appropriately designed substrates or inhibitors. For example, the calculations showed that, at the bottom of the S1 pocket of SC, there was a region of positive potential which could contribute to increased binding strength of a substrate or inhibitor possessing a group of negative potential capable of interacting with this positive enzyme locus. This concept was evaluated with para-substituted phenethylboronic acid inhibitors. Boronic acids of this type are transition state inhibitors of serine proteases that form tetrahedral EI-complexes mimicking the Fig. 4 situation, in which the aromatic group binds in S1. This is illustrated in Fig. 5. In such orientations, the para-substituents should then overlap with the positive region at the base of S1. The results observed support this hypothesis, as shown in Fig. 6, with the stronger binding (reflected by the decreasing Kᵢ values) being manifest as the negative potential character of the para-substituent increases. For the p-chloro-phenethylboronic acid inhibitor, which has the most electronegative para-group, and thus the greatest electrostatic attraction with the positive-potential region beyond the base of S1, binding is 13.5-fold stronger than for the unsubstituted parent compound, phenethylboronic acid. The possibility that the observed trends simply reflected desolvation energy differences between the inhibitors on forming the respective EI-complexes was excluded by literature tabulations of experimental solvation data and by calculations.

Probing stereospecificity

So far, with some exceptions, the use of enzymes in asymmetric synthesis has been largely confined to the creation of chiral synthons with only one stereocentre. However, in principle, the chiral environments provided by enzymes have the capacity to discriminate and control many stereocentres concurrently, potentially providing access to any multiple-stereocentre combination desired in a

Figure 3. Empirical, simple-to-use, active site models, such as shown for PLE, are widely used for predicting and interpreting the structural and stereospecificities of synthetically useful enzymes.

tailor an enzyme’s specificity so that any given structural requirement will be accommodated.

Hydrolytic Enzymes

Probing and modifying structural specificity

Two enzymes that are representative of synthetically useful hydrolases are subtilisin Carlsberg (SC), and chymotrypsin (CT). These serine proteases favour ester substrates possessing hydrophobic groups that bind well into the S1 active site pocket. This is represented schematically in Fig. 4 for the ES-complex formed by SC and its excellent substrate N-acetyl-L-phenylalanine methyl ester (NAPME). In this ES-complex, the hydrophobic amino acid residues that line the S1 pocket create a benevolent environment into which the hydrophobic benzyl group of NAPME fits very nicely. We have carried out several

Figure 4. Schematic representation of the active site of subtilisin Carlsberg, with the non-polar benzyl group of NAPME binding in the hydrophobic S1 pocket. The Gly166 residue is located at the end of S1 where a large region of positive potential begins. The S1' region that accommodates the leaving group contains the serine residue of the catalytically vital Ser221-His64-Asp32 serine protease triad.

Figure 5. Schematic representation of the binding of a boronic acid, transition state analogue, inhibitor to subtilisin Carlsberg.
chiral synthon. We began to explore this question with the two-stereocentre substrates 2 and 3, in which the natural L-configuration preferred by esterases is maintained at the α-amino position, but with either an S (2) or R (3) configuration at C-3. These p-nitro compounds were selected because of their synthetic potential as chloramphenicol precursors. Specifically, the question now became whether or not serine proteases such as subtilisin Carlsberg (SC) or α-chymotrypsin (CT) could discriminate the configuration of the second, C-3, centre.

The kinetic results, together with those on the p-nitrophénylalanyl parent 1 of 2 and 3, showed that the replacement of either the pro-R or pro-S C-3-H by an OH-group caused a >104-fold reduction in the hydrolysis rates for both SC- and CT-catalyzed reactions. While the rates of SC and CT hydrolyses of 2 and 3 were low, they remained preparatively viable. For SC, the hydrolysis rates of 2 and 3 (kcat/KM 126 and 360 M⁻¹s⁻¹ respectively) were about the same, showing that the enzyme did not distinguish significantly between a C-3 S or R centre. Thus separation of a diastereomeric mixture of 2 and 3 could not be achieved using SC catalysis. On contrast, while the rate of CT hydrolysis of 2 was also low (kcat/KM 70 M⁻¹s⁻¹), 3 was a non-substrate. Accordingly, CT could very effectively be applied to separate the individual diastereomers from a mixture of 2 and 3.

The reasons for the severe rate reductions for both SC and CT on introducing a C-3 OH substituent of either configuration, and for the contrasts in the abilities of the two enzymes to distinguish between the two C-3 configurations, were revealed by molecular modelling. The acyl enzyme intermediates derived from the p-nitrophénylalanyl parent compound 1 for each of SC and CT were minimized by molecular mechanics and molecular dynamics using the BioSym Discover program. For the SC-complex, the C-3 hydrogens of 1 were located in the bottom of the S1-pocket in environments about equally restricted, but still able to accept either an S- or R-centre hydroxyl group. Nevertheless, some unfavourable steric interactions are triggered, specifically with Ala152 and Asn155 for a S-centre OH and with Ser 125 for a 3R-centre OH. Thus replacement of either the C-3 pro-S or pro-R hydrogens by hydroxyl, as in 2 or 3 respectively, results in reduced hydrolysis rates for both diastereomers, and to approximately the same degree. Conversely, while the situation for the pro-S C-3-H of 2 in the CT-complex closely resembles that of the SC situation, the pro-R C-3-H of 3 is already in van-der-Waals contact with Cys191, Met192, Gly193 and Asp194. As a consequence, this site cannot accommodate anything bigger than hydrogen. Thus, when the pro-R C-3-H is replaced by OH, as in 3, formation of the acyl enzyme intermediate is precluded, and no hydrolysis of the S,R-diastereomer 3 is can take place. In fact, 3 does not bind at all to CT, as demonstrated by its ineffectiveness as a competitive inhibitor.

As noted above, enzymes have now become generally accepted as chiral catalysts in asymmetric synthesis. However, in almost all cases, including the above, the stereocentre being controlled is closely adjacent to the site of catalysis. Very few examples have been reported where the stereocentre of interest is three or more bonds removed from the carbonyl group of the ester function undergoing hydrolysis. The paucity of examples of stereocentre control remote from enzyme’s catalytic sites parallels the situation in non-enzyme catalyzed asymmetric synthesis, where control of the configurations of stereocentres remote from a chiral auxiliary or catalyst represents a major problem yet to be solved. However, in enzymic catalysis, the whole of an enzyme’s active site region that envelopes a
substrate in the enzyme-substrate(ES) complex is chiral. Thus, in principle, discrimination of any substrate stereocentre is possible, no matter how far from the catalytic site such a stereocentre is located. The abilities of enzymes to discriminate remote stereocentres were probed using SC and CT as representative esterases. SC and CT are commercially available enzymes for which high resolution X-ray crystal structures are available\(^1\) and which have been applied in wide a range of synthetic transformations\(^2\). Each of SC and CT has an extended active site binding region composed of several subsites, of which the S1-pocket dominates, particularly in the binding of hydrophobic groups.

For systematic probing of enzyme specificity, evaluating the binding affinities of transition state analogue competitive inhibitors\(^3\) represents a convenient strategy. Aldehydes are well documented as transition state analogue competitive inhibitors of serine proteases\(^4\). Consequently, the enantiomeric aldehydes \(R\)- and \(S\)-5,7, whose remote stereocentres are positioned either \(\beta\) or \(\gamma\) to the aldehyde, were used to evaluate the remote stereocentre stereoselectivity potential of the S1-pockets of SC and CT, and to further probe the structural specificity of these sites\(^5\). The achiral parent structures 4,6 were also included in the study, as reference compounds.

The inhibitory effects each aldehyde on SC and CT were evaluated\(^6\) using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as substrate. The results, which confirmed each aldehyde to be a competitive inhibitor of both enzymes, are summarized in Fig. 7. Each of the aldehydes 4-7 was a significantly more potent inhibitor of CT than of SC, generally by about an order of magnitude, but by almost two orders of magnitude with \(R\)-5 as the inhibitor. Within each homologous series, binding of an \(R\)-enantiomer is stronger than for either the corresponding \(S\)-enantiomer or its achiral parent, but with the variations being far greater for CT inhibition than for SC. To some degree, this reflects the generally weaker interactions of the inhibitors with SC. The strongest inhibition observed was of CT by \((R)\)-3-phenylbutanal \((R\)-5\), whose \(K_s\) of 8.4 \(\mu\)M is 61-fold lower than that of its achiral parent, 3-phenylpropanol (4), and 88-fold lower than that of its \(S\)-enantiomer. This 88-fold difference in CT-binding affinities between \(R\) and \(S\)-5, demonstrates unequivocally that significant discrimination of remote stereocentres is achievable using enzymes. This is an extremely encouraging result since, if differences in binding of this magnitude were realized between diastereomeric ES-complexes during catalysis of a remote stereocentre-containing racemic substrate, very high resolution efficiencies would be anticipated and the transformation would be of true asymmetric synthetic value. Even the 17.5- and 12.6-fold differences manifest between \(R\)- and \(S\)-5 with SC and \(R\) and \(S\)-7 with CT, respectively, would translate into asymmetric synthetically useful distinctions for analogous remote stereocentre substrates if the overall rates of hydrolysis reflected these levels of binding differences.

Molecular modelling was applied in order to interpret the kinetic data more fully. The X-ray structures of SC and CT were energy-minimized by molecular mechanics and molecular dynamics, and each aldehyde inhibitor was then docked into the active site, with the phenyl moieties in S1, and the aldehyde carbonyl carbon covalently connected to the active site serine-\(\text{CH}_2\text{OH}\) oxygen to form the transition state-like tetrahedral intermediate. Then, each El-complex was energy-minimized, by molecular mechanics, followed by molecular dynamics, calculations, and the minimized El-complexes viewed graphically. Illustrative results for \(R\)- and \(S\)-5 with CT and SC are depicted in Figs. 8 and 9, respectively. The other inhibitors occupied similar active site positions. In the minimized El-complexes of each of 4-7, there were strong interactions between the oxyanion of the tetrahedral intermediate and the oxyanion hole H-
The molecular modelling results on the SC inhibitors are equally informative. The superimposed structures of R- and S-3-phenylbutanal (R- and S-5) in their EI-complexes with SC are depicted in Fig. 9. In both complexes, the phenyl residues of both R-5 and S-5 penetrate adequately, and almost equivalently, into S₁ to provide good hydrophobic binding contributions. For the R-5 orientation, the methyl group at the stereocentre is oriented towards the outside the active site and does not contribute to binding. In contrast to this neutral binding role, the position that the S-5 methyl group is obliged to occupy elicits unfavourable steric interactions of the methyl group with the Asn155 oxyanion hole residue. As a result, the usual oxyanion stabilization mechanism is disturbed. Thus the EI-complex of R-5 with SC is relatively favoured over that of S-5 by the absence of negative interactions rather than, as in the case of CT (Fig. 8), of a beneficial contribution by the methyl substituent.

For the interactions of the higher homologue inhibitors R- and S-4-phenylpentanal (R- and S-7) with SC, the minor (4.8-fold) Kᵢ-variations, and the relatively weak binding of each enantiomer, indicates that there should be little difference in their orientations in the active site, and that are no strong EI-interactions. This is confirmed by the minimized complexes (not shown), in which the phenyl groups of both R- and S-4 are similarly, but not deeply, located in S₁, and with the methyl groups at the stereocentre both oriented away from the active site into locations that do not influence binding.

Comparisons of the ΔΔGᵢ values for the inhibition of CT and SC provide further support for the validity of the molecular modelling interpretations of the weaker R- and
S-stereocentre discrimination for structures 7 than in 5. The \[\Delta \Delta G^f\] values derived from experimental (K_i) data and calculations for the R- and S-pairs of 5 and 7 are similar, each being 50-70\% lower for R- and S-7 than for R- and S-5 for both CT and SC.

The current results represent our first steps towards understanding the factors determining remote stereocentre stereoselectivity of enzymes. Nevertheless, it is clear that the strategy of using competitive inhibitors is an effective one. The observation that the stereocentres of 5 and 7 become less well recognized the more distant they are from the site of reaction parallels the established trend in asymmetric catalysis generally. Thus in attempting to establish enzymic control of the configurations of remote stereocentres, the natural tendency of enzymes to manifest maximum stereoselectivity close to the catalytic site will have to be circumvented. A further objective is to enable guidelines to be formulated that will predict the degrees of stereocentre discrimination, if any, to be expected in the transformations of any new, unnatural substrate structures catalyzed by synthetically useful enzymes. In this regard, the molecular modelling promises to be of value for rapidly screening remote-stereocentre, synthon-precursor, structures of interest, using calculations of their relative energies of binding to appropriate enzymes as a basis for predicting whether or not significant stereocentre discrimination will be attainable.

Inhibiting the Enzymes of Disease: \(\beta\)-Lactamases

The above strategies for probing enzyme specificities are also applicable to the design of inhibitors of enzymes involved in diseases, such as \(\beta\)-lactamases. Due to their key role in the development of bacterial resistance to \(\beta\)-lactam antibiotics, \(\beta\)-lactamases have been of interest for many years and continue to be a topic of intense current interest. Application of \(\beta\)-lactamase inhibitors represents one strategy for overcoming the \(\beta\)-lactam-resistance of these enzymes. The most effective \(\beta\)-lactamase inhibitors described so far are themselves \(\beta\)-lactams, and only a few of these are employed clinically. With the problem of bacterial resistance still increasing, identification of new \(\beta\)-lactamase inhibitors is of considerable clinical interest.

Also, in addition to identifying new lead structures for potential drugs, suitably designed inhibitor studies can help to clarify the mechanistic questions on \(\beta\)-lactamase catalysis that remain. Boronic acid inhibitors have already provided useful information in this regard, for both Class A and C \(\beta\)-lactamases, and have been shown by \(\text{^{11}B-NMR spectroscopy to be reversible transition state analog inhibitors that form tetrahedral adducts with the active site serine of \(\beta\)-lactamases.}

Recently, a 1.7 \AA resolution X-ray structure of the Class A RTEM-1 \(\beta\)-lactamase from *Escherichia coli* was reported. This prompted us to examine the capacities of boronic acids to inhibit this enzyme and to design a transition state analogue inhibitor specifically tailored for \(\beta\)-lactamases, of which the active site characteristics of the RTEM-1 enzyme are typical. The representative cephalosporin substrate-enzyme interactions considered in the rational design process are shown in Fig. 10(a). With the aid of graphics analyses, the boronic acid 8 of Fig. 10(b) was selected as the target inhibitor. For this inhibitor, 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. Finally, the position of the carboxylate moiety selected to elicit strong electrostatic interactions with the Arg 244, Ser 235 and Lys 234 side-chains.

The general synthetic sequence, which is based on the asymmetric synthetic methodology developed by Mattea and coworkers, is shown in Scheme 1. Kinetic evaluation of the boronic acid confirmed that, as designed, it was a highly effective, slow-binding, competitive inhibitor of the Class A RTEM-1 \(\beta\)-lactamase. With its very low K_i of 110 nM, it represents the most potent, transition-state analogue, competitive inhibitor of a \(\beta\)-lactamase yet reported. Its affinity for the enzyme even approaches those of the most powerful mechanism-based inhibitors, such as the penem BRL 42715, and olivamic acid derivative, and is comparable to that of the clinically used clavulanate, sulbactam and tazobactam.

![Figure 10](image-url) (a) Schematic \(\beta\)-Lactamase-cephalosporin Michaelis-complex and (b) of its inhibitor mimic 8.
Scheme 1.

We have also successfully applied similar approaches in the design and synthesis of highly effective conformationally restricted analogue inhibitors of cysteine proteases of medicinal relevance.\textsuperscript{55}

Oxidoreductases

Applications of oxidoreductases with broad specificities, such as HLADH, in asymmetric synthesis have been extensively studied.\textsuperscript{1,2d,26} To complement these broad specificity enzyme data, we therefore decided to examine an oxidoreductase with narrower specificity, with the intent of probing the factors determining structural specificity and stereospecificity. The target enzyme in this report is the L-lactate dehydrogenase of Bacillus stearothermophilus (BSLDH). BSLDH is an excellent vehicle for such studies since it is a very stable, modestly thermophilic, enzyme of known protein sequence and its properties have already been well documented.\textsuperscript{27} In addition, its gene has been cloned, and the protein so efficiently overexpressed that large quantities of BSLDH to be produced inexpensively from small fermentation volumes. Also, the ability to alter the specificity of the native enzyme by site-directed mutagenesis of key active-site amino acid residues has been firmly established.

BSLDH is an NAD/H-coenzyme dependent, fructose-1,6-diphosphate (FPD) activated, enzyme whose in vivo function is to catalyze pyruvate $\leftrightarrow$ L-lactate oxidoreductions of the type:

$$\text{RCOOCOOH} + \text{NAD}^+ + \text{H}^+ \rightarrow \text{RCH(OH)COOH} + \text{NAD}^+$$

While its natural substrate is pyruvate (9, $R = \text{Me}$), with its small $R$-group, BSLDH will accept as substrates a broad range of $\alpha$-ketoacids, but with substantial rate penalties for large or branched $R$-groups. Even so, preparative-scale reactions to produce a range of enantiomerically pure $L$-$\alpha$-hydroxyacids 10 are feasible, as illustrated in Fig. 11.\textsuperscript{28}

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Figure 11. Some illustrative preparative-scale BSLDH-catalyzed reductions of $\alpha$-keto acids.
Modifying structural specificity

Much is known about the structure of the active site of BSLDH\textsuperscript{29}, with the key features being as depicted in Fig. 12. In part, the narrow substrate specificity is due to the fact that the amino acid 98-110 and 235-248 loops close over the ketoacid substrate during the formation of the active ES-complex. This results in an active site of restricted volume that can easily accommodate only small R-side chains. Graphically analyses confirmed that, in a productive ES-complex, large R-groups would elicit bad steric interactions with the loop residue Gln102, but that these adverse interactions with bulky, especially branched-chain, substrates could be ameliorated by reducing the size of the 102 position amino acid side chain. This was tested by using site-directed mutagenesis to replace Gln102 by Asn, an amino acid of similar hydrophobicity but having one fewer ch2-groups in its side chain and thus providing more room for bigger side chains. The experimental data vindicated this approach to structural specificity improvement, with the Gln102Asn mutant being a better enzyme than WT-BSLDH for substrates such as \textit{9}, \( R = \text{CH}_3(\text{CH}_2)_2\text{CH} = \text{CH}(\text{CH}_2)_2\text{CH} = \text{CH}(\text{CH}_2)_2\text{CH} = \text{CH}_2 \), and \( \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \). Furthermore, by replacing Gln102 with the acidic amino acid residues Asp or Glu via site-directed mutagenesis, an active site is created that will now accept positively charged side chains, which the hydrophobic active site of the WT-enzyme normally rejects. When this is done, e-amino-\( \alpha \)-keto acids such as \textit{9}, \( R = -(\text{CH}_2)_2\text{NH}_2 \), that are very poorly accepted by the WT-enzyme, become excellent substrates for the Gln102Asp/Glu mutant BSLDH\textsuperscript{31}. This permits valuable \( \alpha \)-hydroxy-\( \alpha \)-amino acid synthons of considerable biological chiral synthon value to be readily made.

Probing stereospecificity

The stereospecificity of enzymes is their keystone property for asymmetric synthetic applications. However, as noted already, little is known about the factors that determine and control enzyme stereospecificity. Since it is so committed to \textit{Re}-face carboxyl attack to give (\textit{L})-\( \alpha \)-hydroxycis, BSLDH provides an excellent instrument for beginning to identify and understand important stereospecificity determinants, initially of oxidoreductases, but eventually of all enzymes. Among the methods of probing the factors controlling enzyme stereospecificity, evaluating how effectively an enzyme resists attempts to change this capability is potentially one of the most informative. The natural L-stereospecificity of BSLDH is a consequence of the orientation of 2-ketoacids, such as pyruvate (\textit{9}, \( R = \text{CH}_3 \)), in the ES-complex such that the hydrdide-equivalent from NADH is delivered to the \textit{Re}-face of the carbonyl group. This situation is represented, schematically in Fig. 13(a). An key interaction helping to maintain this pyruvate orientation is that between the substrate’s COO\textsuperscript{-} and Arg171. As one gauge of BSLDH’s allegiance to the \textit{L}-pathway, we chose to measure its resistance to being induced to catalyze \textit{D}-lactate formation. Reduction of pyruvate to \textit{D}-lactate requires the “hydride” of the NADH-enzyme to be delivered to the \textit{Si}-face of pyruvate. The simplest way that can be envisaged of inducing this \textit{Si}-face attack would be via an ES complex in which the orientation of pyruvate is “flipped”, as illustrated in Fig. 13(b). Achieving this reversed orientation of pyruvate requires exchanging the natural COO\textsuperscript{-} binding (of Arg171) and hydrophobic, CH\textsubscript{3}-side chain binding (of Gln102 or Ile240) sites by introductions of 171Tyr or 171Trp, or 102Arg or 240Arg/Lys, respectively. Several single and double mutants of these kinds were therefore created.

The stereospecificities of each of the Arg171Tyr and Gln102Arg, Ile240Arg, Ile240Lys, Arg171Tyr/Ile240Arg, Arg171Tyr/Gln102Arg and Arg171Tyr/Ile240Lys mutants were then evaluated for reductions of pyruvate on up to 10 mM scales\textsuperscript{32}, with the NADH-enzyme being recycled using the formate/formate dehydrogenase method\textsuperscript{33}.

The results are summarized in Table 1\textsuperscript{32}. As expected, for WT-BSLDH the product was exclusively \textit{L}-lactate, with no \textit{D}-lactate whatsoever being detectable. The same situation prevailed for the Ile240Arg and Ile240Lys single mutants, for which the natural Arg171-COO\textsuperscript{-} lock was still operational. However, the Arg171Tyr and Gln102Arg mutations began to disturb the stereochemical fidelity of BSLDH in exactly the way for which they had been designed, with 0.2% and 0.6% respectively of \textit{D}-lactate being produced. For the double mutant Arg171Tyr/Gln102Arg,
Figure 13. Schematic representation of pyruvate bound at the active site of BSLDH in orientations leading to \( L \)- or \( D \)-lactic acid formation. In (a) the natural binding mode that results in \( L \)-lactate formation is depicted. Part (b) illustrates the “flipped” binding orientation of pyruvate envisaged to be inducible by mutations such as Arg171Tyr, Gln102Arg, and combinations thereof. Such mutations effectively reverse those of the natural pyruvate binding sites and should therefore lead to \( D \)-lactate as the product.

### Table 1. Percentages of \( D \)-lactate produced on catalysis of pyruvate reduction (on a 10 mM-scale using the Fig. 11 methodology) by BSLDH and its mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>% D-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (I240/R171)</td>
<td>0</td>
</tr>
<tr>
<td>Ile240Arg</td>
<td>0</td>
</tr>
<tr>
<td>Ile240Lys</td>
<td>0</td>
</tr>
<tr>
<td>Arg171Tyr</td>
<td>0.2</td>
</tr>
<tr>
<td>Gln102Arg</td>
<td>0.6</td>
</tr>
<tr>
<td>Arg171Tyr/Gln102Arg</td>
<td>1.1</td>
</tr>
<tr>
<td>Ile240Arg/Arg171Tyr</td>
<td>0.5</td>
</tr>
<tr>
<td>Ile240Lys/Arg171Tyr</td>
<td>2.3</td>
</tr>
</tbody>
</table>

with its greater potential for binding pyruvate in the “flipped”, Fig. 13(b), orientation, the proportion of \( D \)-lactate formed was still higher, at 1.1%.

The Ile240Lys/Arg171Tyr double mutant was the most successful in terms of the degree of stereospecificity reversal achieved, with a very significant 2.3% of \( D \)-lactate formed. This represents a truly remarkable switch in stereospecificity-preference, from a \( D \)-enantiomer formation-frequency of \(< 1 \) in 25,000 for WT-BSLDH to 1 in 43 for the Ile240Lys/Arg171Tyr mutant. This represents an experimentally demonstrated favouring of the \( D \)-pathway of at least 500-fold. The lower limit of sensitivity of our analytical method is ± 0.004%. Thus any levels of \( D \)-lactate formations in reductions of pyruvate catalyzed by WT-BSLDH is certainly below this threshold. The actual integrity of the \( L \)-stereospecificity of \( L \)-LDH’s is certainly much higher than is possible to measure by our current assay method. In this regard, it is notable that LaRoue and Anderson 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. 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 truly dramatic > 2 x 10^5 -fold relaxation of the \( L \)-stereospecificity of the double mutant enzyme relative to that of WT-BSLDH. However, it is evident that there is a network of secondary “fail-safe” interactions which BSLDH can invoke to maintain the substrate in its natural orientation when the preferred stereochemical determinants are removed. The remarkable tenacity of such fail-safe interactions is reflected by the fact that even the most successful Ile240Lys/Arg171Tyr double mutant is still > 97% \( L \)-stereoselective. Interestingly, the “reversed” substrate binding mode strategy appears to be the way that Nature has adopted in producing \( D \)-lactate, since the active sites of \( L \)- and \( D \)-LDH’s seem to be virtual mirror images of one another.

The stereospecificity probing results are particularly exciting since the partial reversals of stereocentre configuration were achieved on an enzyme that is of the most highly committed stereospecificity. Thus, for other synthetically useful enzymes whose stereospecific control
mechanisms are more relaxed, especially towards unnatural substrates, the application of protein engineering to control and tailor a desired stereospecificity outcome now becomes a realistic goal.

Concluding Perspective

The data presented in this perspective clearly represent only a beginning towards identifying the factors that determine enzyme specificity, it is evident that much needs to be done before enzymes and substrates can be tailored with confidence to permit optimum results in all asymmetric synthetic applications, and to maximize their performance and stability as catalysts. Nevertheless, very soon the tremendous progress now being made in protein research is certain to reveal many new insights into enzyme catalysis, and on how to control and modify specificity. Such knowledge will stimulate unprecedented expansion of the asymmetric synthetic uses of enzymes. Furthermore, it must be recognized that, so far, the only enzymes in synthetic use are those catalyzing common metabolic reactions. In the future, with the aid of pathway engineering and directed evolution methods, it will become possible to tap the enormous additional resources that the total microbial and plant genomes contain. For example, within these genomes there are undoubtedly many "silent" genes which code for currently unnecessary enzymes. When these genes are identified, and activated, the scope of enzyme catalysis will expand still further. The recent discovery of a Diels-Alderase\(^{36}\), which fills a previously missing gap in synthetic enzyme methodology, provides just one illustration of what can be expected from this untapped potential for new enzymes awaiting discovery.

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References


