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Review Article

Probing Enzyme Specificity†

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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. In view of the increasingly broad spectrum of unnatural substrate structures that synthetically useful enzymes are required to handle, it is becoming essential to delineate the enzyme–substrate interactions that regulate structural specificity and stereospecificity. This will then permit the identification of the enzymes best suited to transforming new substrate structures into the desired chiral synthons. Such knowledge of the factors controlling and determining optimum active site binding and orientation of any potential substrate structure will also facilitate the tailoring of enzyme specificity by protein engineering. Some results of initial studies probing the specificities of esterases and oxidoreductases of synthetic value, and of modifying their properties by site-directed mutagenesis, are described.

Enzymes are now broadly accepted as useful catalysts for a broad range of organic synthesis, with their capacities for inducing asymmetric transformations being the most exploited.[1] However, despite the widespread uses of both enzymes and microorganisms in asymmetric synthesis, relatively little is known of the factors that determine the structural specificity and stereospecificity of enzymes. In view of the increasingly broad spectrum of new and unnatural substrate structures that synthetically useful enzymes are being called on to accommodate, it is becoming more and more essential to delineate the enzyme–substrate interactions that regulate and control enzyme specificity. This will then permit the selection of the enzymes that are best suited for any given chiral synthon preparation. It will also facilitate the development of active site models capable of accurately forecasting whether an enzyme will accept a new structure as a substrate, and of reliably predicting what the stereochemical outcome of the reaction will be. In addition, knowledge of the factors determining specificity will aid the rational tailoring of enzyme specificity by the site-directed mutagenesis techniques of protein engineering.

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The classes of enzymes most widely applied are the hydrolases and the oxidoreductases and this perspective will focus on probing the specificity of representative, synthetically useful, members of both of these groups.

Strategy

Because of their simplicity and ease of application, active site specifications of the Fig. 1-type[1,2] are presently the 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 understanding of enzyme specificity that in the long term is essential for identifying the range of substrate structures any synthetically useful enzyme can accept, and for selecting the most appropriate enzyme for transforming a given substrate structure into a desired synthon. Accordingly, in order to gain insights into the factors that control and determine enzyme specificity, we have begun to probe the nature of enzyme–substrate interactions in a systematic manner. This involves studying synthetically useful enzymes for which good X-ray structures are available, using graphics analyses to select substrate or inhibitor structures that address a particular specificity question most appropriately and, after kinetic studies on the selected struc-

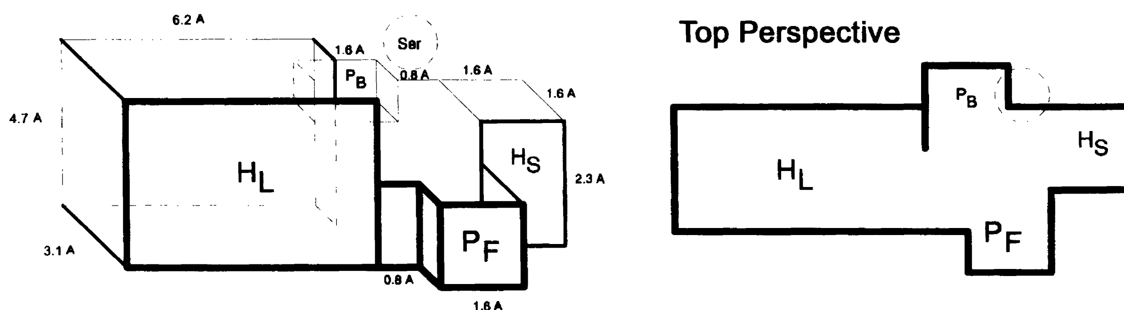


Fig. 1. Empirical, simple-to-use, active site models, such as this one for pig liver esterase,[2] are widely used for predicting and interpreting the specificities of synthetically useful enzymes towards chiron–precursor substrates.

tures, analyzing the experimental data with the aid of graphics and molecular modelling methods. The eventual goal of this approach is to maximize the synthetic potential of each enzyme. However, no natural enzyme can be expected to handle the ever increasing range of substrate structures imposed by the chiral synthon demands of asymmetric synthesis. An important corollary of this strategy is thus its potential for identifying 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 correct unfavourable amino acid positions, and eventually to tailor an enzyme's specificity so that any given structural requirement will be accommodated.

Probing the structural specificity of hydrolases

The target enzymes selected as being representative of synthetically useful hydrolases were subtilisin Carlsberg (SC, EC 3.4.21.14), and α-chymotrypsin (CT, EC 3.4.21.1). These serine proteases favour ester substrates possessing hydrophobic groups that bind well into the S_1[3] active site pocket, as represented schematically in Fig. 2 for the ES-complex formed by SC and its excellent substrate *N*-acetyl-L-phenylalanine methyl ester (NAPME). In this ES-complex, the hydrophobic benzyl group of NAPME fits nicely into the S_1 pocket, which provides a welcoming environment as a result of the hydrophobic amino acid residues that line it. We have done a number of studies delineating and modifying the structural specificities of the S_1 sites of serine proteases,[4] of which the most recent have been directed towards evaluating electrostatic effects.[5]

When the electrostatic potential surfaces of SC and CT were calculated (using the BioSym *Delphi* programme), the patterns for the two enzymes were seen to be very different. We wondered whether such 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 S_1 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. The initial evaluations of this concept were carried out with *para*-subtituted phenethylboronic acid inhibitors. When bound to serine proteases, boronic acids of this type are transition state inhibitors[6] that form tetrahedral EI-complexes mimicking the Fig. 2 situation, in which the aromatic group binds in S_1. In such orientations, the *para*-substituents should then project into the positive region at the base of S_1. The results observed[5] support this concept, as shown in Fig. 3, with the strength of binding increasing (reflected by the decreasing K_I values) as the negative potential character of the *para*-substituent increases. For the *p*-chlorophenethylboronic acid inhibitor, which has the most electronegative *para*-group and thus the greatest electrostatic attraction with the positive-

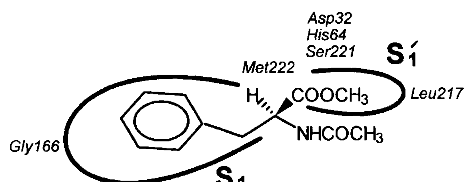


Fig. 2. Schematic representation of the active site of subtilisin Carlsberg, with the non-polar benzyl group of NAPME binding in the hydrophobic S_1 pocket. The Gly166 residue is located at the end of S_1 where a large region of positive potential begins. The S_1' region that accommodates the leaving group contains the serine residue of the catalytically vital Ser221-His64-Asp32 serine protease triad.

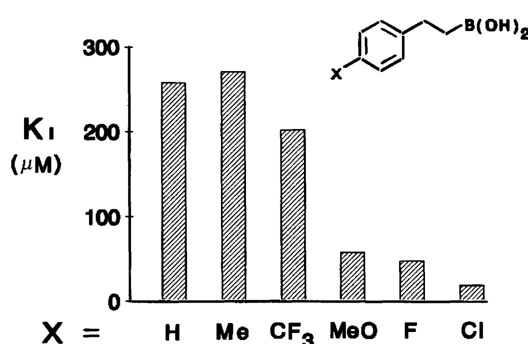


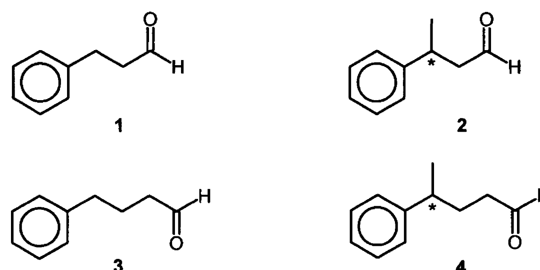
Fig. 3. Electronegative groups in the *para*-position of phenylboronic acid inhibitors of Subtilisin Carlsberg increase binding power.

potential region beyond the base of S_1, binding is 13.5 times 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 calculations and from literature tabulations of experimental solvation data.

Probing hydrolase stereospecificity

As noted already, enzymes have now gained general acceptance as chiral catalysts in asymmetric synthesis.[1] However, in almost all cases, the stereocentre being introduced or selected is 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.[7] 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 that has not yet been solved.[8] However, in enzymic catalysis, since the whole of an enzyme's active site region that envelopes a substrate in the enzyme–substrate (ES) complex is chiral, discrimination of any substrate stereocentre is feasible in principle, no matter how remotely such a stereocentre is located from the catalytic site. These questions regarding the abilities of enzymes to discriminate remote stereocentres was addressed, using SC and CT as representative hydrolases. SC and CT are commercially available enzymes that have been applied in wide a range of synthetic transformations[9] and for which high resolution X-ray crystal structures are available.[10] These serine proteases have an extended active site binding region composed of several subsites, of which the S_1-pocket dominates, particularly in the binding of hydrophobic groups.

For systematic probing of enzyme specificity, evaluating the binding affinities of transition state analog competitive inhibitors[4b,5] represents a convenient strategy. Aldehydes are well known to be transition state analog competitive inhibitors of serine proteases.[11] Accordingly, the enantiomeric aldehydes *R*- and *S*-**2**,**4**, with remote stereocentres positioned either β or γ to the aldehyde, together with the achiral parent structures **1**,**3** as reference compounds, were used to evaluate the remote stereocentre stereoselectivity potential of the S_1-pockets of SC and CT, and to further probe the structural specificity of these sites.[12]



The inhibitory effects of each aldehyde on SC and CT were evaluated by the method of Waley,[13] using Suc-Ala-Ala-Pro-Phe-PNA as substrate. The results, which showed that each aldehyde was a competitive inhibitor of both enzymes, are summarized in Fig. 4.

Each of the aldehydes 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*-**2** as the inhibitor. Furthermore, 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. In part, this reflects the generally weaker interactions of the inhibitors with SC. The strongest inhibition observed was of CT by (*R*)-3-phenylbutanal (*R*-**2**), whose K_I of 8.4 μM is 61 times lower than that of its achiral precursor, 3-phenylpropanal (**1**), and 88 times lower than that of its *S*-enantiomer. This 88-fold difference in CT-binding affinities between *R*- and *S*-**2**, with its unequivocal demonstration that significant discrimination of remote stereocentres is achievable using enzymes, is an extremely

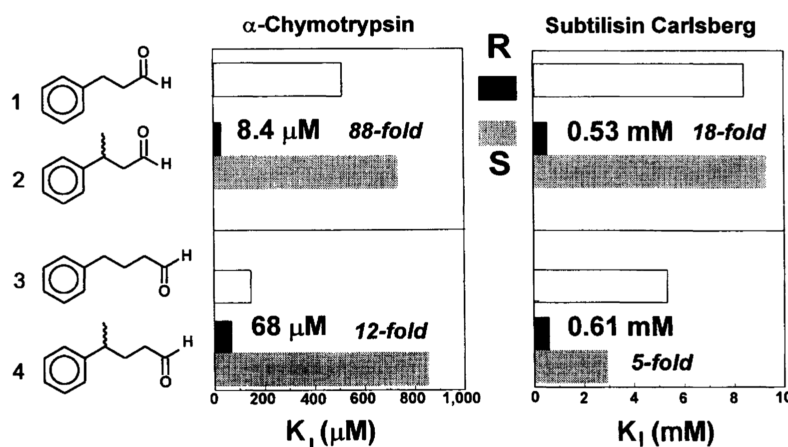


Fig. 4. Inhibition of Subtilisin Carlsberg and α-chymotrypsin by the aldehydes **1–4**.

encouraging result. For example, 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*-**2** with SC and *R*- and *S*-**4** 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. Each aldehyde inhibitor was then docked into the active site, with the phenyl moieties in S_1, and the aldehyde carbonyl carbon covalently connected to the active site serine-CH_2OH oxygen to form the transition state-like tetrahedral intermediate. Each EI-complex was then subjected to energy-minimization, by molecular mechanics, followed by molecular dynamics, calculations, and the optimized conformations and the free energies of each *R*- and *S*-pair of EI-complexes compared. The results for the chiral inhibitors are depicted in Figs. 5–8. The achiral inhibitors also occupied very similar active site positions. In each minimized EI-complex, there were strong interactions between the oxyanion of the tetrahedral intermediate and the oxyanion hole H-bonding residues of the peptide backbone NHs of Ser195 and Gly193 of CT, and the backbone NH of Ser221 and the side chain NH_2 of Asn155 of SC, respectively.

The superimposed conformations of (*R*)- and (*S*)-3-phenylbutanal (*R*- and *S*-**2**) in the active site of CT (Fig. 5) show that the methyl group at the stereocentre of the *R*-enantiomer is positioned in the S_2 pocket such as to elicit favourable hydrophobic interactions with the enzyme. In contrast, the corresponding methyl group of the *S*-enantiomer is located outside of the active site of the enzyme and is oriented towards the external water in a manner that does not contribute to hydrophobic bonding. In addition, the phenyl group of the *R*-enantiomer is able to locate itself deeper inside the hydrophobic S_1-pocket than can its enantiomeric equivalent, thereby providing a stronger phenyl–S_1 contribution to binding. It is the combination of these two factors that accounts for the dramatic 88-fold difference in K_I between *R*-**2** and *S*-**2** as inhibitors of CT. In the cases of the homologous inhibitors, (*R*)- and (*S*)-4-phenylpentanal (*R*- and *S*-**4**), the much smaller (12.6-fold) K_I differences between inhibition of CT by the enantiomers are also accounted for by the molecular modelling results (Fig. 6). The superimposed structures of *R*-**4** and *S*-**4** in the active site of CT reveal that the methyl groups of both the *R*- and *S*-enantiomers are directed towards the outside of the active site. The *S*-enantiomer is obliged to adopt this unusual orientation in order to avoid unfavorable steric interactions between the methyl group and the side chain of Met 192. As a consequence, this prevents the phenyl group of *S*-**4** from penetrating as deeply into the hydrophobic S_1-pocket as it might otherwise, thereby precluding the strong phenyl–S_1 hydrophobic interactions that *R*-**4** enjoys. The latter enantiomer does not bind as strongly as *R*-**2** above because it lacks the additional methyl–S_2 interaction manifest in Fig. 5.

The molecular modelling results on the SC inhibitors are equally revealing. The superimposed structures of (*R*)-and (*S*)-3-phenylbutanal (*R*- and *S*-**2**) in their

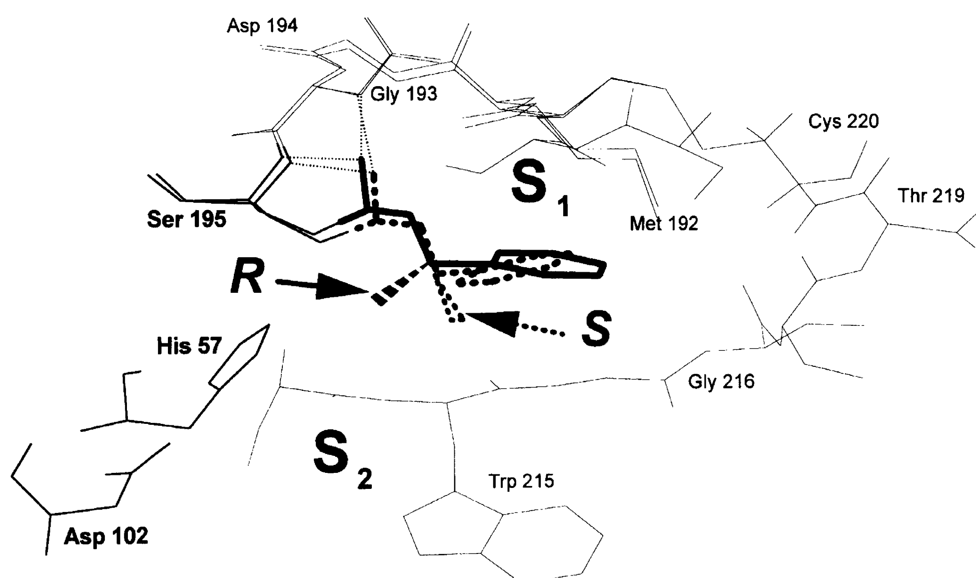


Fig. 5. Superimposed energy-minimized EI-complexes of *(R)*- and *(S)*-3-phenylbutanal, *R*-**2** (—) and *S*-**2** (------), respectively, in the active site of CT. The main difference between the two orientations is that the methyl group at the stereocentre of *R*-**2** (—▸) is favorably located in the S_2 pocket, while that of *S*-**2** (······>) is oriented towards the outside of the active site and does not contribute to binding.

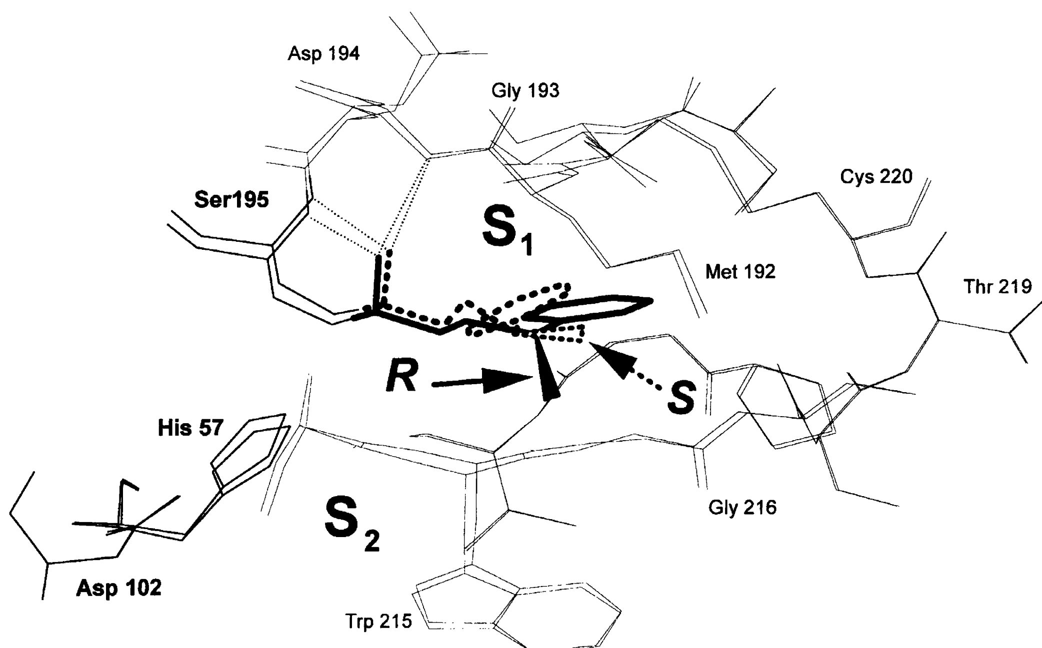


Fig. 6. Superimposed energy-minimized EI-complexes of (*R*)- and (*S*)-4-phenylbutanal, *R*-**4** (—) and *S*-**4** (------), respectively, in the active site of CT. The stereocentre-methyl groups of both *R*-**4** (—▸) and *S*-**4** (······>) are obliged to locate outside the active site and are not binding contributors. The main differences are in the locations of the phenyl groups, with that of *R*-**4** being positioned the more deeply in the hydrophobic S_1.

EI-complexes with SC are depicted in Fig. 7. In both complexes, the phenyl residues of both *R*-**2** and *S*-**2** penetrate adequately, and almost equivalently, into S_1 to provide good hydrophobic binding contributions to the corresponding K_Is. For the *R*-**2** orientation, the methyl group at the stereocentre is oriented towards the outside the active site and does not make any binding contribution. In contrast to this neutral binding role, the position that the *S*-**2** methyl group is obliged to occupy elicits unfavorable steric interactions of the methyl group with the Asn155 oxyanion hole residue. As a result, the usual oxyanion stabilization mechanism is disturbed. Thus the

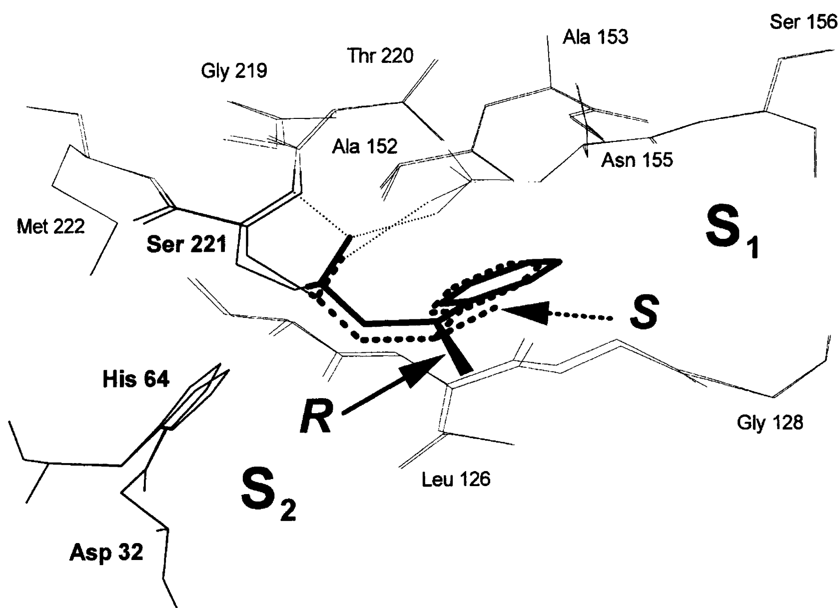


Fig. 7. Superimposed energy-minimized EI-complexes of (*R*)- and (*S*)-3-phenylbutanal, *R*-**2** (—) and *S*-**2** (------), respectively, in the active site of SC. Both phenyl residues bind in S_1. The stereocentre-methyl group of *R*-**2** (—▸) is oriented outside of the active site where its binding influence is neutral, while that of *S*-**2** (······>) takes up a position close to Asn155 and is responsible for the disruption in the oxyanion stabilization.

EI-complex of *R*-**2** with SC is relatively favoured over that of *S*-**2** by the absence of negative interactions rather than, as in the case of CT (Fig. 5), of a beneficial contribution by the methyl substituent.

For the interactions of the higher homolog inhibitors (*R*)-and (*S*)-4-phenylpentanal (*R*- and *S*-**4**) with SC, the minor (4.8-fold) K_I-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 there are no strong EI-interactions. This is confirmed by the minimized complexes of Fig. 8, in which the phenyl groups of both *R*- and *S*-**4** are similarly, but not deeply, located in S_1, and with the methyl groups at the stereocentre both oriented away from the active site into locations that do not influence binding.

A comparison of $\Delta\Delta G\ddagger$ values for the inhibition of CT and SC provides further support for the validities of the molecular modelling analyses of the weaker *R*- and *S*-stereocentre discrimination for structures **4** than in **2**. The differences in experimental (from K_Is) and calculated (from EI-complex energies) $\Delta\Delta G^{\ddagger}$s for the *R*- and *S*-pairs of **2** and **4** are similar, each being 50–70% lower for *R*- and *S*-**4** than for *R*- and *S*-**2** for both CT and SC.

While the current results represent only a first step towards probing remote stereocentre stereoselectivity of enzymes, the strategy of using competitive inhibitors is clearly an effective one. The observation that the stereocentres of **2** and **4** become less well recognized the more distant they are from the site of reaction is in accord with the established trend in asymmetric catalysis generally.[8] Thus in attempting to establish enzymic control of the configurations of remote stereocentres, the natural proclivity of enzymes to manifest maximum stereoselectivity close to the catalytic site will have to be conquered. Another of our ultimate goals is to formulate guidelines for forecasting the degrees of stereocentre discrimination to be expected in the transformations of any new, unnatural substrate structures catalyzed by synthetically useful enzymes. In this regard, 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.

Oxidoreductases

Applications of oxidoreductases with broad specificities, such as horse liver alcohol dehydrogenase (HLADH), in asymmetric synthesis have been widely studied.[1] 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 and stereospecificity. The enzyme selected was the L-lactate dehydrogenase of *Bacillus stearothermophilus* (BSLDH). BSLDH is an excellent candidate for such exploratory 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.[14] Also, its gene has been cloned and the protein very efficiently overexpressed, thereby enabling large quantities of BSLDH to be produced inexpensively from small fermentation volumes. Furthermore, the

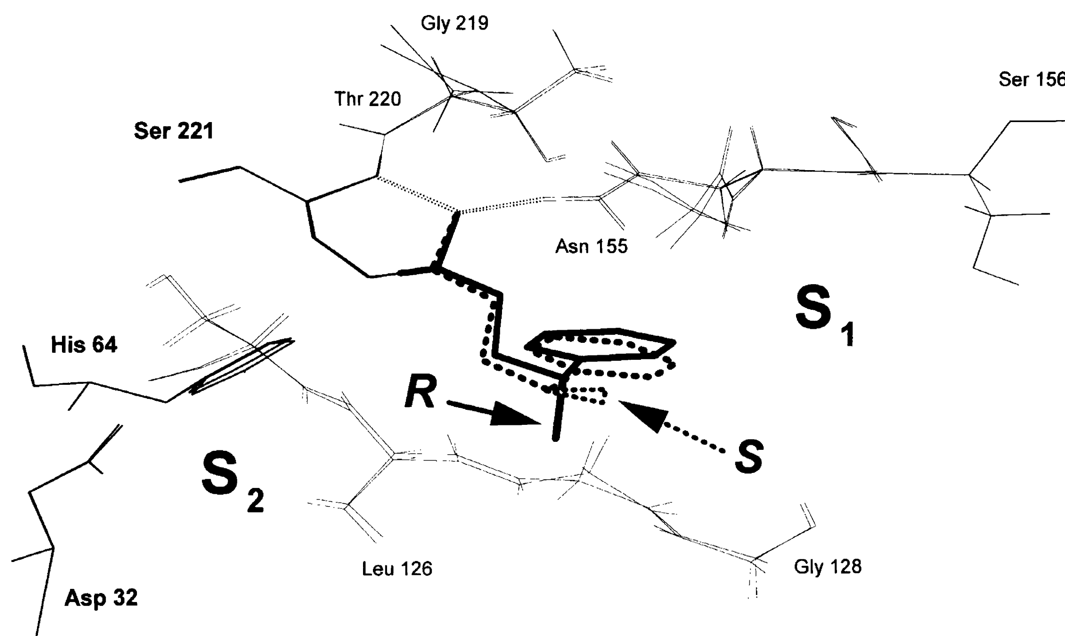
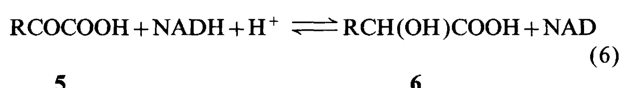


Fig. 8. Superimposed energy-minimized EI-complexes of (*R*)- and (*S*)-4-phenylbutanal, *R*-**4** (—) and *S*-**4** (------), respectively, in the active site of SC. The methyl groups at the stereocentres of *R*-**4** (—→) and *S*-**4** (······>) are both located outside of the active site and are non-contributors to binding. The only minor difference between the two EI-complexes is that the phenyl group of *R*-**4** penetrates more deeply into the hydrophobic S_1 region than does that of *S*-**4**.

feasibility of altering the specificity of the native enzyme by site-directed mutagenesis of key active-site amino acid residues has been established.

On the structural specificity of BSLDH

BSLDH is an NAD/H-coenzyme dependent, fructose-1,6-diphosphate (FDP) activated enzyme, whose *in vivo* function is to catalyze pyruvate$\rightleftharpoons$L-lactate oxido-reductions of the type (1).



$$\underset{\mathbf{5}}{RCOCOOH} + NADH + H^+ \rightleftharpoons \underset{\mathbf{6}}{RCH(OH)COOH} + NAD \quad (6)$$

While its strongly preferred substrate is pyruvate (**5**, R = Me) with its small *R*-group, BSLDH will accept as substrates a broad range of α-keto acids, albeit with substantial rate penalties for large or branched R-groups. Nevertheless, preparative-scale reactions to produce a range of enantiomerically pure L-α-hydroxy acids **6** are feasible.[15]

Much is known about the structure of the active site of BSLDH.[16] The key features are represented in Fig. 9. The narrow substrate specificity is due, at least in part, to the fact that the 98–110 and 235–248 loops close over the keto acid substrate during the formation of the active ES-complex, thereby leaving only a restricted volume for the R-side chains. Graphics analyses revealed that, in the productive ES-complex, large R-groups would engender bad steric interactions with the loop residue Gln102 and indicated that these adverse interactions with bulky, especially branched-chain, substrates could be diminished by reducing the size of the 102-position amino acid side chain. The validity of this analysis was tested by using site-directed mutagenesis to replace Gln102 by Asn, an amino acid of similar hydrophobicity but having one fewer CH_2-groups in its side chain and thus providing more room for bigger side chains. The results obtained supported the application of such protein engineering approaches to expand the structural specificity of enzymes, with the Gln102Asn mutant now being a better enzyme than WT-BSLDH for substrates such as **5**, $R = CH_3(CH_2)_{2-5}$, $(CH_3)_2CH$, $(CH_3)_2CHCH_2$, $CH_3CH_2CH(CH_3)$, and C_6H_5.[17] 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. When this is done, ε-amino-α-keto acids such as **5**, $R = (CH_2)_{1-2}NH_3^+$, that are very poorly accepted by the WT-enzyme, then become excellent substrates for the Gln102Asp/Glu mutant BSLDHs.[18]

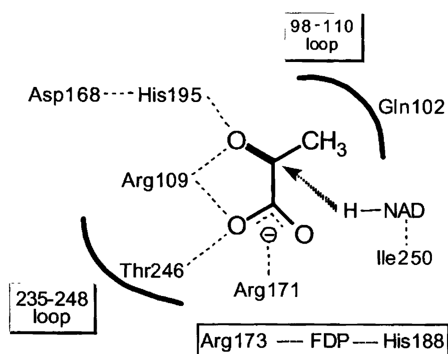


Fig. 9. Schematic representation of some of the important active site residues of BSLDH involved in catalysis and binding and their interactions with pyruvate (based on the X-ray data of Wigley *et al.*[16a] for the BSLDH·oxamate·NADH·FDP complex). The dashed lines show the pyruvate-carboxylate interactions with Arg171 and Arg109, and of His195 and Arg109 with the pyruvate-carbonyl group. The direction of hydride delivery to the *Re*-face of the pyruvate-carbonyl that leads to L-lactate formation is shown by the hatched arrow. The closing and opening of the 98–110 loop is the rate determining step for the natural reaction.

Probing BSLDH stereospecificity

The stereospecificity of enzymes is their most important property for asymmetric synthetic applications. However, as noted already, little is known about the factors that determine and control enzyme stereospecificity. With L-LDHs being so committed to *Re*-face carbonyl attack to give L-α-hydroxy acids, 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 powerful. The natural L-stereospecificity of BSLDH is determined by the orientation of 2-keto acids, such as pyruvate (**5**, $R = CH_3$), in the ES-complex such that the hydride-equivalent from NADH is delivered to the *Re*-face of the carbonyl group. This is depicted schematically in Fig. 10(a). An important interaction helping to maintain this pyruvate orientation is that between the substrate's COO^- and Arg171. As one measure of BSLDH's commitment to the L-pathway, we elected to evaluate its resistance to being induced to catalyze D-lactate formation. Reduction of pyruvate to D-lactate requires delivery of the NADH-'hydride' to the *Si*-face of pyruvate. One of the ways that can be envisaged of inducing this *Si*-face attack would be via an ES complex in which the orientation of pyruvate was reversed, as illustrated in Fig. 10(b). Achieving this 'flipped' orientation of pyruvate requires swapping the natural COO^--binding (of Arg171) and hydrophobic, CH_3-side chain binding (of Gln102 or Ile240) sites by introductions of 171Tyr or 171Trp, or 102Arg or 240Arg/Lys, respectively. Various single and double mutants of these kinds were therefore created.

The stereospecificities of the Arg171Tyr and Gln102Arg, Ile240Arg, Ile240Lys, Arg171Tyr/Ile240Arg, Arg171Tyr/Gln102Arg and Arg171Tyr/Ile240Lys mutants were then individually evaluated for reductions of pyruvate on up to 10 mM scales,[19] using formate/formate dehydrogenase recycling of the NAD/H coenzyme.[20] The

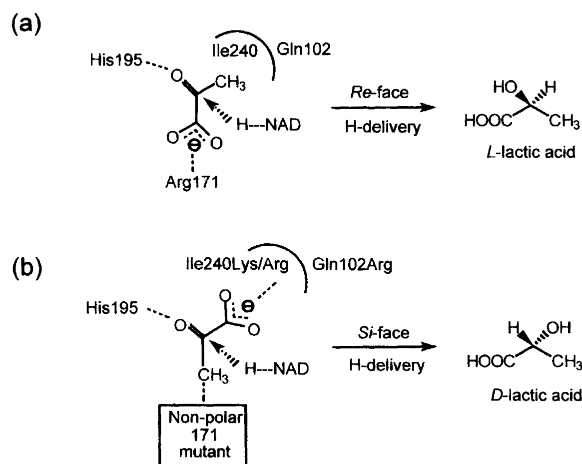


Fig. 10. Schematic representation of pyruvate bound at the active site of BSLDH in orientations leading to L- or D-lactic acid formation. Part (a) represents the natural binding mode that results in L-lactate formation. Part (b) illustrates a reversal of the natural binding orientation of pyruvate that might be induced by mutations such as Arg171Trp/Tyr, Gln102Arg, and combinations thereof, that would 'flip' the pyruvate binding sites and lead to D-lactate as the product.

results are recorded in Table 1.[19] As expected, for WT-BSLDH the product was exclusively L-lactate. No D-lactate whatsoever could be detected. The same was true for the Ile240Arg and Ile240Lys single mutants, for which the natural Arg171-COO^- lock was still operational. However, the Arg171Tyr and Gln102Arg mutations did begin to disturb the stereochemical integrity of BSLDH in the very manner for which they had been designed, with 0.2% and 0.6%, respectively, of D-lactate being produced. For the double mutant Arg171Tyr/Gln102Arg, with its greater potential for binding pyruvate in the reversed, Fig. 10(b), mode, the proportion of D-lactate formed was higher still, at 1.1%.

The Ile240Lys/Arg171Tyr double mutant was the most successful in terms of the extent of stereospecificity reversal, with a very significant 2.3% of D-lactate produced. This represents a truly remarkable stereospecificity-preference switch, from a D-isomer formation-frequency of <1 in 25 000 for WT-BSLDH to 1 in 43 for the Ile240Lys/Arg171Tyr mutant, which represents an experimentally measurable improvement in the favoring of the D-pathway of at least 500-fold. The lower limit

Table 1. Percentages of D-lactate produced on catalysis of pyruvate reduction by BSLDH mutants.

Mutant	% D-Lactate
WT (I240/R171)	0
Ile240Arg	0
Ile240Lys	0
Arg171Tyr	0.2
Gln102Arg	0.6
Arg171Tyr/Gln102Arg	1.1
Ile240Arg/Arg171Tyr	0.5
Ile240Lys/Arg171Tyr	2.3

of sensitivity of our analytical method is $\pm 0.004\%$, and any D-lactate formation in reductions of pyruvate catalyzed by WT-BSLDH is certainly below this level. The actual L-fidelities of L-LDHs are certainly much higher than possible to measure by our current assay method. The elegant studies of LaReau and Anderson[21] have demonstrated that for NAD^+, non-stereospecific 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 fidelity of the associated hydride transfer from NADH to pyruvate is comparable. Based on this parallel, the 2.3% D-lactate production observed with Ile240Lys/Arg171Tyr represents a truly dramatic $>2 \times 10^5$-fold relaxation of the L-stereospecificity of the double mutant enzyme relative to that of WT-BSLDH. Nevertheless, it is clear that there is a network of secondary 'fail-safe' interactions which BSLDH can invoke to maintain the substrate in its natural orientation.[22] The remarkable tenacity of such interactions is reflected by the fact that even the most successful double mutant Ile240Lys/Arg171Tyr is still $>97\%$ L-stereoselective.

It should be noted that there are other serendipitous benefits to the 171 mutations of the above studies in that the thermal stability of BSLDH is dramatically increased for the Arg171Tyr enzyme, whose apparent melting temperature is 9.4 °C higher than that of WT-BSLDH. The greatest increase in apparent melting temperature was 10.7 °C for Arg171Tyr, Gln102Arg. This means that the latter mutant retains 30% of its activity even after being heated for 30 min at 100 °C. Under such conditions, the already moderately thermostable WT-enzyme is completely inactivated in less than 2 min.[23] These increases in thermal stability may have considerable potential synthetic benefits since preparative-scale BSLDH-catalyzed reactions at temperatures of up to 100 °C can now be contemplated, subject to NAD-coenzyme survival for sufficient time. The reason for the increased thermal stabilizations on replacing the Arg171 by hydrophobic residues such as Trp seems due to more favourable hydrophobic subunit contacts.[24]

The stereospecificity probing results are particularly encouraging since the partial configuration reversals were achieved on an enzyme of the most highly committed stereospecificity. Thus, for other synthetically useful enzymes whose stereochemical control mechanisms are less restrictive, particularly towards unnatural substrates, the exploitation of protein engineering to tailor and control desired stereospecificity now becomes a realistic possibility. In this regard, it is of interest to consider how Nature controls L- vs. D-LDH stereospecificity. No D-LDH structure is yet available but X-ray diffraction studies are presently being carried out on several enzymes. In the meantime, an indication of Nature's possible strategy may be obtained from examination of the structure of the *Hyphomicrobium methylovorum* D-glycerate dehydrogenase (GDH),[25a] an enzyme closely homologous to D-LDH and whose X-ray coordinates are

available from Brookhaven Protein Data Bank.[25b] D-LDHs and GDH catalyze the same kind of reaction, and operate on similar substrates, except that the natural substrate for GDH is hydroxypyruvate.

To compare L-LDH and GDH active site architectures, we superimposed, via graphics, the active site residues of BSLDH and GDH. The results of this modelling are depicted in Fig. 11. The proton-donating nitrogens of the imidazolium rings of each active site histidine are located in similar, almost overlapping, positions close to the carbonyl oxygen of the pyruvate equivalent oxamate, as required for catalysis. However, the most exciting observation is that the active site arginines of GDH and BSLDH are located on *opposite* sides of the substrate, in an almost mirror-image relationship. It thus appears that the strategy adopted in our probing of stereospecificity reflects the one that Nature herself selected for controlling the stereochemistry of L- and D-LDH-catalyzed reductions of 2-keto acids.

The same conclusion regarding the mirror-image relationship between D- and L-LDH active site geometries was reached recently by Lamzin and coworkers,[26] and in a modified manner by Goldberg *et al.*[25a] More recently, calculations by the group of Feytmans[27] have indicated that in D-LDHs, the critical COO^--binding Arg residue is not quite in an L-mirror position, but is somewhat displaced to permit hydrogen-bonding to pyruvate's carbonyl group. The recently completed[28] D-LDH X-ray studies by Pai and coworkers provide experimental support for this latter prediction.

While the data presented in this perspective represent 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. However, in the next few years, the tremendous progress now being made in protein research is certain to lead to many exciting new insights into enzyme catalysis, and on how to control and modify specificity. Such information will permit unprecedented expansion of the asymmetric synthetic uses of enzymes. Furthermore, up to now, the only enzymes being exploited are those catalyzing common metabolic reactions. In the future, with the aid of pathway engineering and directed evolution methods, it will become possible to exploit the enormous additional resources that the total microbial and plant genomes represent. These genomes undoubtedly contain many 'silent' genes which code for currently unnecessary enzymes. When these genes are identified, and turned on, the scope of enzyme catalysis will undoubtedly include virtually all the reaction types of synthetic value.

The recent discovery of a Diels–Alderase[29] is just one illustration of filling a previously missing gap in synthetic enzyme methodology, and represents just the 'tip-of-the-iceberg' of what may be expected from this this untapped potential for new enzymes.

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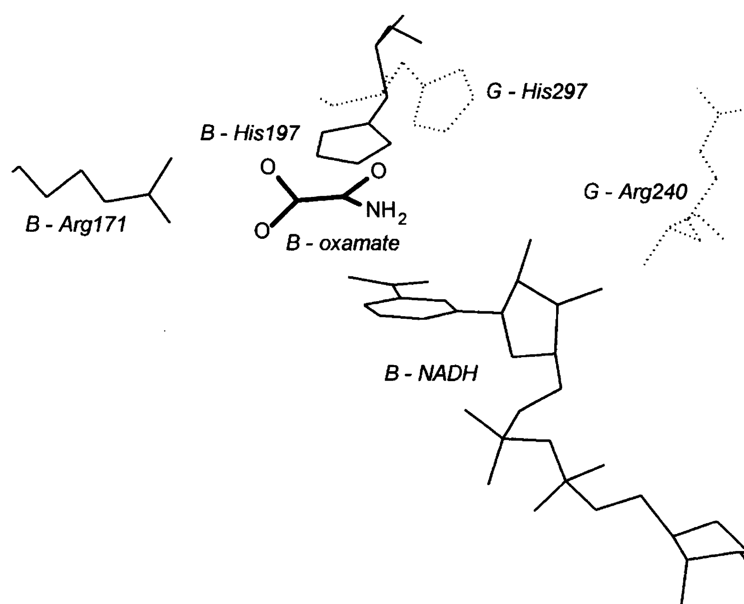

Fig. 11. Superposition of the GDH and BSLDH active sites. Only the histidine residues involved in carbonyl polarization and protonation and the carboxyl-binding arginine residues are depicted. The B- and G-prefixes denote residues from BSLDH (solid lines) and GDH (dotted lines), respectively. The NADH-binding site is in the common domain. The catalytically important histidine residues of both enzymes are in almost equivalent locations. In contrast, the carboxylate-binding arginine residues of the two enzymes are seen to be on opposite sides of the substrate, with an approximate mirror plane passing through the COO^- group of oxamate (the pyruvate equivalent) and the histidine imidazolium groups.

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