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Esterases in organic synthesis: present and future

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Abstract - The current, and potential future, applications of esterases in organic synthesis are illustrated by some aspects of pig liver esterase-catalyzed reactions and the development of an active site model of predictive value for this enzyme, together with examples of the controlled modification of subtilisin specificity by protein engineering using the site-specific mutagenesis techniques of molecular biology.

INTRODUCTION

The abilities of enzymes to act as chiral catalysts for a broad spectrum of organic synthetic reactions has been known for many years (ref. 1). The field is now well documented (ref. 2) and the use of enzymes in synthesis has become accepted as a powerful and unique complement to the traditional chemical methods of the organic chemist's synthetic arsenal. Hydrolytic enzymes are among the most widely used enzymes, particularly in asymmetric synthesis, with esterases of the serine protease type having been especially widely applied. It is on two members of this group that attention will be focused. Firstly, some aspects of the synthetic applicability of pig liver esterase, an enzyme of considerable current utility, will be considered. Secondly, some approaches to the controlled modification of the properties of subtilisin, a less widely applied enzyme to date, will be presented. Subtilisin will be used to provide initial examples of how we can hope to tailor the specificity of enzymes in a predetermined manner using the site-specific mutagenesis techniques of molecular biology. This will have great impact in the future for the creation of superior enzyme catalysts for a broad range of organic synthetic applications.

PIG LIVER ESTERASE

Although very little is known about the structure or amino acid sequence of PLE, it has proven a phenomenally successful catalyst in asymmetric synthesis (ref. 2). However, the enzyme is not without its problems. One worry that has emerged is that PLE is not constant in its stereoselectivity within a given series of compounds. For example, for the series of meso cycloalkane-1,2-dicarboxylic acid diester substrates, PLE-catalyzed hydrolysis is stereospecific for the S-centre ester for the cyclopropanyl- and cyclobutanyl-substrates, but becomes R-centre specific for the cyclohexanyl diester. The cyclopentane compound represents the change-over structure, with both ester groups being hydrolyzed to similar extent to give the acid-ester product of only 17%ee. The acid-esters so produced are valuable chiral synthons, as exemplified by the ready access they provide to enantiomeric series of derivatives via selective chemical transformations of either the carboxyl or ester function (ref. 3a). Another concern up till now has been that PLE-stereoselectivity is apparently fickle (ref. 3) with, for example, acyclic 3-substituted glutarate diesters being hydrolyzed with what appears at first sight to be random stereoselectivity (ref. 3c).

Another potential drawback of PLE-catalyzed generation of chiral synthons has been that the ee's of the products are sometimes too low for asymmetric synthetic purposes. For example, PLE-catalyzed hydrolysis of dimethyl 3-methylglutarate under the normal pH7 aqueous conditions gives the (3R)-methyl glutarate monoester product of only 77%ee. This can be considered the result of having two competing diastereomeric enzyme-transition state complexes that differ in activation energy by 0.8kcal/mole under the room temperature conditions of hydrolysis. In order to obtain a 95%ee product, this energy difference needs to be expanded to 2kcal/mole - an energy change of only 1.2kcal/mole. This can be achieved

by changing the reaction conditions so that the thermodynamic and/or kinetic constants of the two pathways are affected differently, and to induce changed conformations of the protein so that the enzyme-transition state complex energies are altered in the right direction. At present, this must be done empirically by varying the reaction temperature, ionic strength, nature and concentrations of organic solvents, etc. until the pathway-energy separations leading to the desired ee's are achieved. Thus, when the above glutarate hydrolysis is carried out at -10°C in 20% methanol of apparent pH7, the product ee becomes fully acceptable at 97%.

The hitherto unpredictable nature of PLE stereoselectivity has also been resolved by the formulation of a comprehensive and reliable active-site model for the enzyme, that is of predictive value. In creating this model, we built on our own (ref. 3c) and other (refs. 4a-c) previous proposals , and also analyzed all PLE-specificity results in the literature. By combining these data into a computer graphics analysis, the simple four-site binding picture depicted in Fig. 1 emerged. In order to be hydrolyzed, the ester group of a substrate must locate adjacent to the serine nucleophile of the enzyme (cf Fig. 1). Of the four pockets shown, two $P_{F(ront)}$ and $P_{B(ack)}$ are polar in nature, the other two are hydrophobic, one $H_{L(arge)}$ being much bigger than the other, $H_{S(mall)}$. In order for an ester to be a substrate, it must be able to fit into these regions appropriately, with polar and hydrophobic moieties binding into complementary sites. The stereoselectivity-determining factor is whether or not a hydrophobic group fits into H_S or H_L. Clearly, if a group is forced to swing from H_S-binding to H_L-binding, it will rotate the substrate orientation and change from an R to S (or vice versa) preference. This is the basis for PLE's stereoselectivity changes, and the reasons for its previously fickle behaviour are now fully rationalized. All literature substrate-behaviours fit this model. Space does not permit a description of its application, but is fully described in ref. 5. Current work is now being directed at delineating fully the characteristics of the H_L, H_S, P_F, P_B pockets.

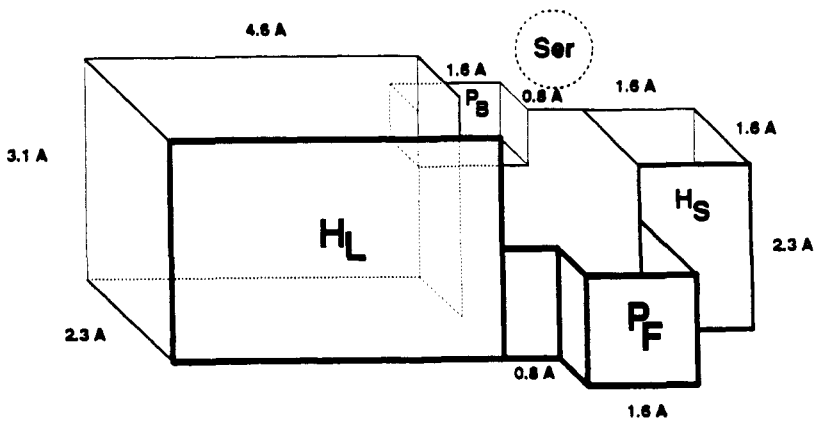

Figure 1. Active-site model for pig liver esterase.

SUBTILISIN

For PLE, an incompletely characterized enzyme of unknown structure, the above empirical analyses and manipulations represent the only approach to understanding and rationalizing its specificity. In contrast, for subtilisin BPN', the detailed structural and mechanistic knowledge available presents a vast horizon of opportunities for control. Subtilisin BPN' is a serine protease of known X-ray structure (ref. 6a) that has been cloned and subjected to site-specific mutagenesis (ref. 6b) This situation opens up almost infinite possibilities for tailoring the properties of an enzyme to suit any natural or unnatural substrate structure of synthetic interest. While protein engineering along these lines is still only at the very beginning, it represents one of the frontiers at which the future potential of enzyme applications is being developed. The following results represent some first steps in tailoring the specificity of subtilisin. This work was done in collaboration with the Genencor "team" led by David Estell using Genencor mutant enzymes and X-ray structures (ref. 6).

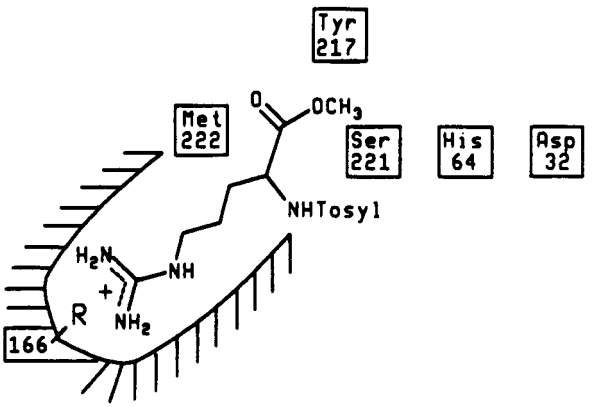

Fig. 2. Schematic representation of the active site and binding pocket (P_1) of subtilisin BPN'.

Subtilisin prefers to bind hydrophobic residues in its P_1 pocket (Fig. 2). In order to see whether P_1 could be made more receptive to polar groups, the kinetics of hydrolysis of N-tosylarginine methyl ester (TAME) was studied (Table 1). With the wild-type enzyme, which has glycine at amino acid position 166 of the P_1 pocket, TAME binds less well (higher K_m) than when Gly166 is replaced by asparagine, whose $-CH_2CONH_2$ side chain is able to attract TAME more strongly by hydrogen-bonding to its guanidinium group. However, the intrusion of the relatively large $-CH_2CONH_2$ function into the P_1 volume disturbs the orientation of TAME, as reflected by its low k_{cat} value. For the Ser166 enzyme with its $-CH_2OH$ side chain, a good compromise between its size (for easy accommodation in P_1) and hydrogen-bonding capability (to the guanidinium of TAME) is reached, with good k_{cat} and K_m values, and the best specificity constant (k_{cat}/K_m) of the series. Graphics analyses confirm that the Ser166 mutant is optimal for TAME binding and hydrolysis.

TABLE 1. Kinetic constants for wild-type and mutant subtilisin-catalyzed hydrolyses of N-tosylarginine methyl ester (TAME).

AA166	R	k_{cat} (s^{-1})	K_M (M x 10^{-3})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Gly	-H	5.4	34	160
Asn	$-CH_2CONH_2$	0.39	21	19
Ser	$-CH_2OH$	2.7	14	190

The specificity of the leaving group pocket can also be altered rationally. When the natural methionine 222 residue (Fig. 2) is replaced by phenylalanine, graphics analysis shows that there is less volume available in this site. This should reduce the catalytic efficiency of the Met222→Phe enzyme towards substrates with a large leaving group interaction in the rate determining step, and is found to be the case experimentally. Tyrosine 217 acts as a "wall" at the end of the leaving group pocket and restricts the volume of the leaving group that can be accommodated. This negative effect should be somewhat alleviated by replacing Tyr217 with a smaller residue such as leucine, and, in fact, catalysis of substrates with large leaving groups such as para-nitroanilide by the Tyr217→Leu mutant is much faster than for the wild-type enzyme.

These experiment represent our first steps towards developing an understanding of the factors determining enzyme specificity and in its control. Our eventual goal, and that of others in the field (ref. 7) is to develop the capability to create custom catalysts for any substrate transformation that is needed in organic and asymmetric synthesis. While we have a long way to go before this can be achieved, I am confident that the protein engineering approach will have dramatic impacts on the use of enzymes in organic synthesis in the near future.

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