

the immobilized enzyme has several advantages over the use of the soluble one; it allows shorter reaction times because of the higher concentration of the enzyme, the final yield (98%) is higher, and the work-up is much easier because the biocatalyst can be removed by simple filtration.

We thank Dr. P. Armisen for the Ni²⁺-IDA-agarose gift. Dr. A. Vian is acknowledged for the gift of the pAG plasmid. We are indebted to Dr. A. Fernández-Mayoralas for his continuous encouragement and helpful discussions. A.B. was supported by a postdoctoral I3P contract of the European Social Fund. This work was supported by the Spanish Dirección General de Investigación (Grants BQU2001–1503 and PTR1995–0568-OP).

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Received: October 23, 2002

Revised version: March 7, 2003 [Z514]

Selective Protein Degradation by Ligand-Targeted Enzymes: Towards the Creation of Catalytic Antagonists

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KEYWORDS:

affinity cleavage · enzymes · protein design · receptors · selectivity

The selective degradation of proteins by proteinases is an integral regulatory process that supplements and complements the regulation of expression-controlled protein concentrations. Recently several regulatory proteinase systems have been implicated in functions as diverse as the pathogenesis of Alzheimer's disease^[1] or arteriosclerosis^[2] through to the facilitation of synaptic processes in the brain.^[3] Proteinase-activated receptors constitute a family of G-protein-coupled receptors that are uniquely activated, or indeed deactivated, through partial degradation by extracellular serine proteinases.^[4, 5]

The likelihood of the existence of many more unknown regulatory proteinases has highlighted the need for probes that will explore therapeutic potential.^[6] Although the selective destruction of proteins by proteinases is a widespread process in nature, the design of de novo degradative systems that can uniquely accomplish elimination or control of the function of a preselected specific target protein has provided a formidable challenge. Pioneering work by Wilchek and co-workers^[7] in 1990 explored the possibility of avidin–biotin induced affinity cleavage of streptavidin protein targets by proteinases bearing biotin as a targeting ligand. However, this work showed that indiscriminately biotinylated proteolytic enzymes were not only unable to cleave the avidin protein target but were even less

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active towards avidin than unbiotinylated enzyme.^[7] Thus, uncontrolled positioning of multiple biotin-targeting ligands created an unwanted blocking rather than an enhancing effect.^[8]

We report here examples of chimeric proteinases that successfully target and degrade preselected proteins by using appropriate low-molecular-weight ligands (the targeting ligand) that bind to the protein (the target) at its functionally relevant site. In this strategy, the appropriate ligand acts as a homing device to confer and enhance selectivity of up to over 350-fold in a generic process that exploits the intrinsic, ligand-recognition capabilities of the protein target to trigger its own destruction. In this two-component process, the targeting ligand moiety first acts analogously to a classical antagonist to bind the target protein, and the target protein is then catalytically degraded by the attached proteinase moiety (Figure 1 a). Since there are already well-documented and readily-available ligands suitable for use as targeting ligands for many proteins, this strategy allows facile design of, and access to, such chimeric proteinases without the need for extensive protein re-engineering for each new application. The net effect, shown here for three important protein classes, is to create powerful molecules that approach the ideal of catalytic antagonists (CAs), that is, enzymes that selectively destroy protein function.

Chimeric proteinases consisting of a targeting ligand covalently-linked at or near to the substrate-binding site of the

proteinase were constructed. This was achieved by introducing a cysteine residue at a preselected position by site-directed mutagenesis, followed by chemical modification of the newly-introduced thiol side chain with MTS reagents^[9] to create chemically modified mutant enzymes^[10, 11] (Figure 1 a). The resulting chimeric proteinase construct combines the advantages of the binding ability of low-molecular-weight antagonists with the catalytic degradative hydrolytic activity of proteinases. Accordingly, these constructs may be viewed as ligand-targeted proteinases that act as CAs.

For our study, we selected the serine proteinase subtilisin from *Bacillus lentus*^[12] (EC 3.4.21.62, formerly EC 3.4.21.14) as the most suitable demonstration enzyme model. SBL displays functional similarity to regulatory proteinases and indeed is a member of the same S8 peptidase family as the regulatory serine proteinase, subtilisin convertase furin.^[13, 14] In addition, SBL has been well characterized,^[15] possesses a broad substrate specificity, its crystal structure is known,^[16] and, importantly for our purposes, it contains no natural cysteine residues. Highly thiol-selective MTS reagents therefore react only with the cysteine residue that is introduced by mutagenesis.

Five active-site-region attachment sites for the targeting ligand were selected and surveyed: Asn62, Ser101, Ser156, Ser166, and Leu217 (Figure 1 b). Collectively, these allowed us to position the targeting ligand at different points around the radius of the active site "mouth" of the CA. Each of these wild-

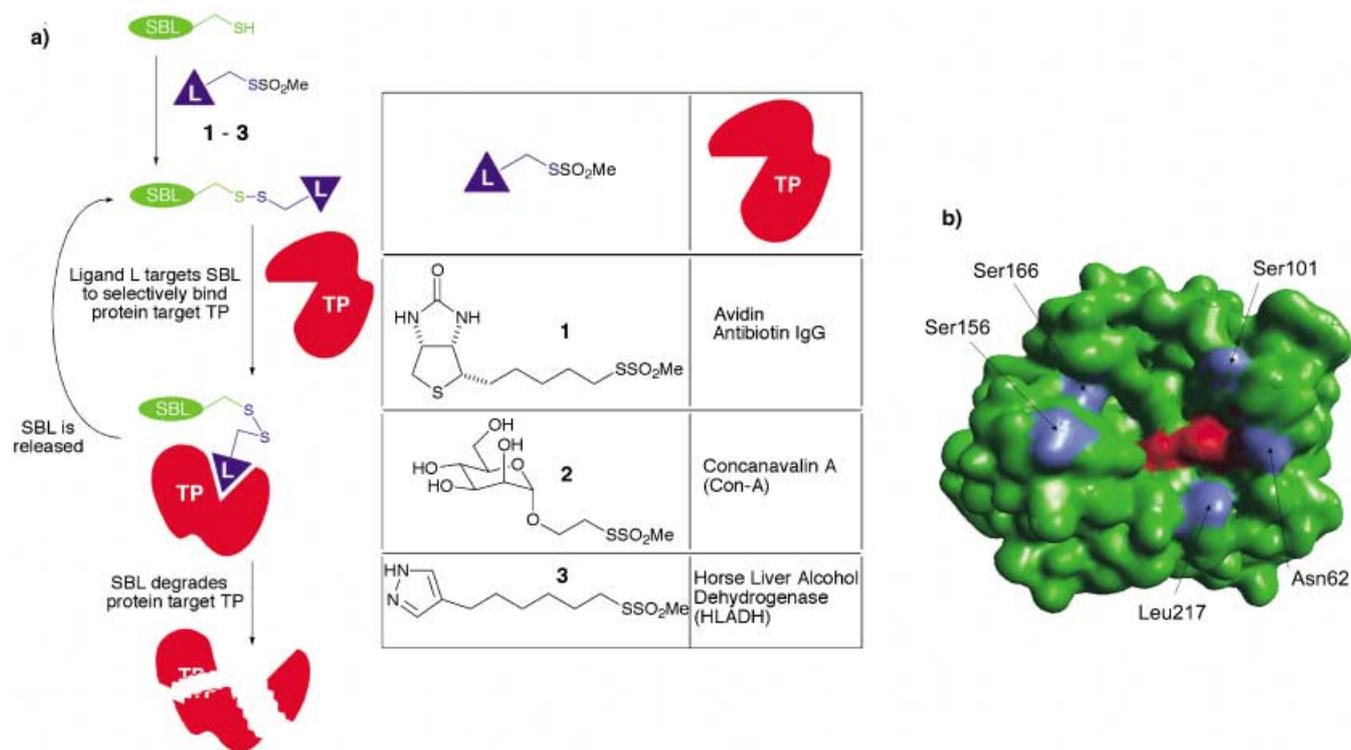


Figure 1. a) The concept of a catalytic antagonist (CA) and its creation. The thiol group of single-point cysteine subtilisin (from *Bacillus lentus*; SBL) mutants is chemoselectively modified with targeting ligand (L) by using the appropriate methanethiosulfonate (MTS) reagent (1–3 in this study) to give a CA. For example, L-MTS + S156C subtilisin can be used to create CA S156C-L. The target protein (TP) binds L at its complementary site, thereby juxtaposing the TP and the active site of the SBL chimera. By virtue of this proximity, enhanced CA-catalyzed hydrolytic degradation of TP ensues. The structures of the MTS reagents, which contain natural ligands or inhibitors, used to introduce L and their corresponding TPs are shown in the box; b) The five positions (blue), Asn62, Ser101, Ser156, Ser166, and Leu217 in SBL selected for attachment of targeting ligands. The residues that form the catalytic triad, Asp32, His64, Ser221, are colored red.

type (WT) residues was replaced by a cysteine residue. Then SBL-based CAs bearing targeting ligand moieties attached at each of these positions were created by using the MTS compounds 1–3 to modify the introduced cysteine thiol group in the desired manner (Figure 1 a). In this way, CAs designed to target a binding protein (avidin), a lectin (concanavalin A, con-A), an antibody (IgG), and an enzyme (horse liver alcohol dehydrogenase, HLADH) were created. All of the CAs prepared were active proteinases, with values of k_{cat}/K_M (catalytic rate constant/Michaelis constant) towards the standard small substrate amide sucAAPFPNA in the range 22.7–115.0 $\text{s}^{-1}\text{mM}^{-1}$, compared with 209 $\text{s}^{-1}\text{mM}^{-1}$ for the WT enzyme.

We chose avidin, with its strong affinity for the small ligand biotin, as a representative binding protein target. In addition, the prior difficulties in enhancing protein degradation encountered by Wilchek and co-workers with biotin as a targeting ligand^[7] made this a suitably challenging benchmark system. Biotin–MTS (1, Figure 1 a) was used to introduce the biotin targeting ligand to prepare the corresponding biotinylated CAs. HABA–avidin (HABA = 2-(4'-hydroxyazobenzene)benzoic acid) titration^[17] with the resulting CAs demonstrated that S156C-biotin had the highest affinity for avidin (apparent dissociation constants, K_D : S156C-biotin, $1.1 \times 10^{-15}\text{M}$; N62C-, S166C-, L217C-biotin, 1.1 – $1.4 \times 10^{-13}\text{M}$). The ability of these CAs to degrade avidin correlated well with this affinity. S156C-biotin (Figure 2 a) gave the most pronounced results, with a 5.2-fold higher rate of degradation of the protein target avidin compared to untargeted, native SBL. This degradation pattern for large proteins contrasts sharply with earlier studies^[18] in which modification at position 156 had little or no effect on intrinsic, small substrate specificities. All biotinylated CAs degraded avidin more rapidly (1.3- to 5.2-fold enhanced degradation) than untargeted WT SBL. However, consistent with its high affinity for its target, S156C-biotin CA displayed the highest selectivity for the target over a decoy. In the presence of an equimolar concentration of decoy protein (disulfide scrambled RNase-A), the parent WT SBL showed a 2.6-fold increase in degradation fragment peptide release rate relative to the amount seen when only avidin was present in the assay solution. This increase in peptide fragment concentration was attributed to additional peptidic material from decoy degradation.^[19] This result was qualitatively confirmed by a Western blot probed with antiribonuclease-A. In contrast, a virtually negligible increase was observed for the avidin-targeted S156C-biotin CA in the presence of an equimolar concentration of decoy, or even when challenged with a fivefold excess of decoy. Taken together, the increase in activity (5.2-fold) and the very small relative rate of decoy peptide degradation for S156C-biotin ($0.014 \times$ rate of degradation of untargeted WT-SBL) represent a selectivity enhancement of more than 350-fold. The enhanced degradative activity of S156C-biotin was unequivocally confirmed by using polyacrylamide gel electrophoresis (PAGE) time course monitoring (Figure 2 b). The actions of untargeted WT-SBL and S156C-biotin were also compared. In the presence of S156C-biotin, marked degradation of the avidin band (20000 MW) was observed within 15 min. Corresponding densitometry data from the gel confirmed similar rate enhancements to those observed by monitoring low-molecular-weight

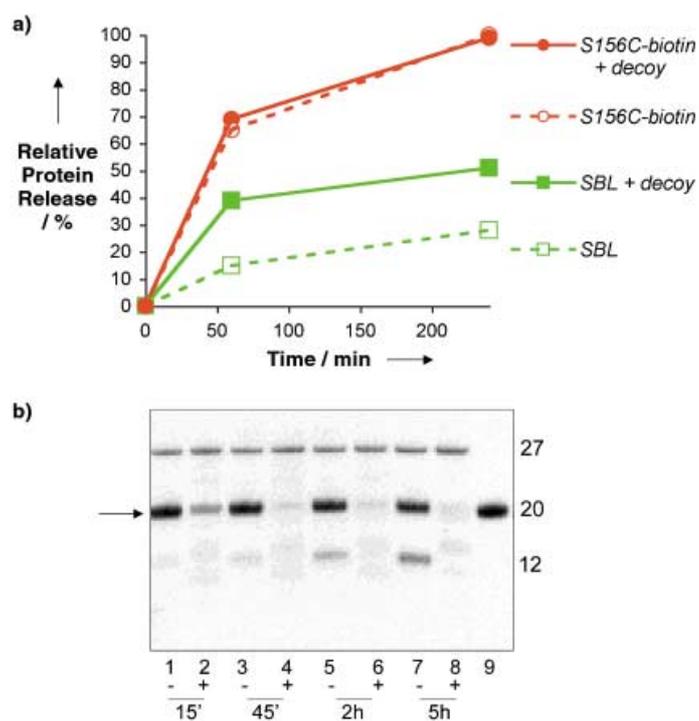


Figure 2. Targeted avidin destruction as measured by (a) peptide production and (b) SDS PAGE. a) The degradation of avidin by targeted S156C-biotin (red) or untargeted WT SBL (green) as determined by the creation of protein fragments (< 3000 MW) as a function of time in the absence (open symbols and dashed lines) or presence (closed symbols and solid lines) of decoy protein (disulfide scrambled RNase-A). b) Degradation of avidin in the presence of untargeted SBL or S156C-biotin as a function of time. After incubation for 15 min, 45 min, 2 h, and 5 h, samples were inhibited with phenylmethylsulfonyl fluoride then boiled in the presence of SDS and subjected to SDS-PAGE. The upper band (27000 MW) is untargeted WT SBL (–) or S156C-biotin (+), while the lower band (20000 MW) indicated with an arrow is that of the avidin target (Lane 9 = avidin control). Elimination of the avidin band is seen for samples incubated with S156C-biotin and little or no diminution of the avidin band is detected for even the longest incubation with untargeted WT SBL (Lanes 1–8).

fragments. In contrast, the avidin treated with untargeted WT-SBL was virtually unaffected even after 5 h.

To explore the ability of this method to retarget the same proteinase SBL to another binding protein target, we equipped the proteinase with a D-mannose targeting ligand by using D-mannose-MTS (Compound 2).^[20] The S156C-mannose CA created showed enhanced degradation of its new protein target, the mannose-binding lectin concanavalin A. In this case, the somewhat lower levels of enhancement of degradation (only 1.3-fold enhanced over untargeted WT SBL) and more modest levels of selectivity (1.5-fold) that were observed compared to the system with avidin were in accord with the lower affinity that lectins display for single monosaccharide ligands (typical $K_D \sim 10^{-3}\text{M}$).

Immunoglobulin targeting was then examined. Selective reduction of levels of particular immunoglobulins is an attractive approach to the treatment of allergenic responses^[21,22] and for the prevention of hyperacute rejection following xenotransplantation.^[23] The biotinylated CAs described above were readily extended to the targeting of a model monoclonal anti-biotin-IgG. Enzyme-linked immunosorbent assay (ELISA)^[24] was used to

evaluate the binding of each biotinylated CA. As for avidin targeting, the S156C-biotin CA had the highest affinity for IgG ($K_D \sim 4 \times 10^{-8} \text{ M}$). Moreover, the targeted degradative activity of S156C-biotin towards anti-biotin-IgG was similar to that seen for the degradation of avidin. Both with and without decoy protein, the degradation of anti-biotin-IgG was enhanced 3.5-fold. Pretreatment of anti-biotin-IgG with biotin prior to the addition of S156C-biotin CA yielded intact IgG with preserved binding function. This protective effect of biotin provided confirmation that biotin binding to the recognition site of the target is indeed the primary targeting interaction of this CA.

We next evaluated the abilities of CAs to target and destroy a single enzyme in the presence of other enzymes or proteins. As the molecular targets for most current small-molecule therapeutics, enzymes arguably represent the most therapeutically immediate class of protein targets and consequently offer the widest range of potential targeting ligands. We chose horse liver alcohol dehydrogenase as a model enzyme target, and its competitive inhibitor, 4-hexylpyrazole (inhibition constant, $K_i \sim 10^{-8} \text{ M}$),^[25] as the targeting ligand. 4-Hexylpyrazole-MTS (**3**) was used to precisely construct N62C-, S101C-, S156C-, S166C- and L217C-pyrazole CAs. Consistent with our other results (see above), the S156C-pyrazole CA ($K_D \sim 10^{-10} \text{ M}$) was the most effective HLADH-degrading CA. The redox activity of HLADH was used as an excellent measure of protein functional integrity. While HLADH function decreased by only 12% in 3 h in the presence of untargeted WT SBL (Figure 3 a), HLADH activity was more severely reduced (88% loss of activity) in the same time period in the presence of the targeted S156C-pyrazole CA.

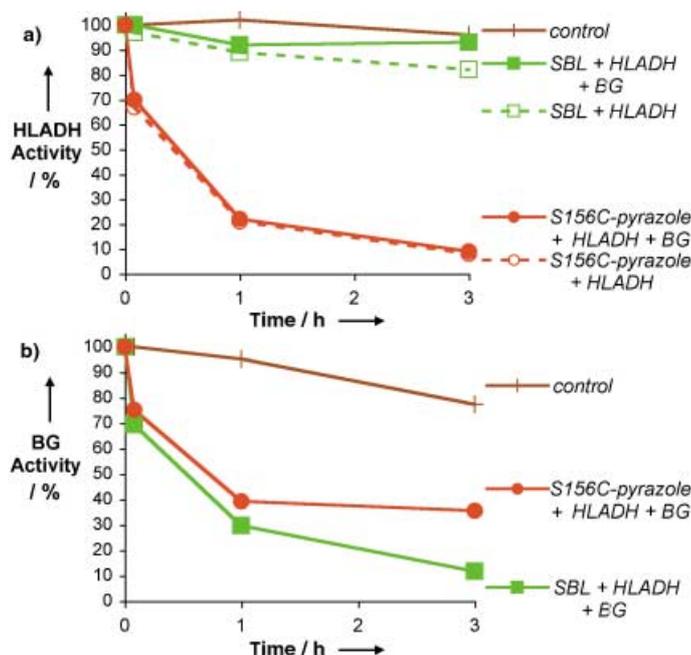


Figure 3. Enhanced destruction of HLADH. a) The degradation of enzymatic activity of HLADH alone (+ HLADH, dashed lines and open symbols) or in a mixture with a second, decoy enzyme BG (+ HLADH + BG, solid lines and closed symbols) by targeted S156C-pyrazole (red) or untargeted WT SBL (green). b) The enzymatic activity of BG in a two-protein mixture of HLADH + BG degraded by targeted S156C-pyrazole (red) or untargeted WT SBL (green).

To evaluate the degree of selectivity of this enhanced degradation in the presence of other enzymes, these experiments were repeated with a mixture of the HLADH target and carbonic anhydrase.^[26] Over 3 h, HLADH was again nearly completely degraded, while carbonic anhydrase was unaffected. Since, carbonic anhydrase is inherently highly resistant to proteolysis we also decided to evaluate a much more sensitive, and therefore testing, decoy enzyme. Accordingly, we evaluated β -galactosidase (BG) from bovine liver as a more challenging decoy,^[26] as it is a far less robust enzyme and is therefore a more facile substrate for untargeted WT SBL.^[27] This greater susceptibility of BG to degradation by SBL was confirmed in the protein mixture (Figure 3 b) and, correspondingly, the hydrolysis of HLADH by untargeted WT SBL in the presence of the decoy BG, was simultaneously reduced (Figure 3 a). From this result we conclude that BG is a more attractive substrate than HLADH for untargeted WT SBL and that, in the two-enzyme mixture untargeted WT SBL opportunistically hydrolyzes BG as a competing substrate in preference to HLADH. Despite this intrinsically biased situation, incubation of the same mixture with targeted S156C-pyrazole CA still shows a significant increase in the overall degradation of HLADH relative to untargeted WT-SBL (88% degraded after 3 h as compared with 3% for untargeted WT SBL; Figure 3 a). It is noteworthy that the S156C-pyrazole CA not only degrades HLADH to a much greater extent, but at the same time shows reduced degradation of BG (63% of the degradation achieved by untargeted WT SBL) compared to untargeted WT-SBL (Figure 3 b).^[28] Taken together, the enhanced HLADH degradation (29.3-fold) and reduced BG degradation ($0.63 \times$ degradation by untargeted WT-SBL) represents a 45-fold overall enhancement in selectivity with the CA.

The current results represent a significant advance in ligand-targeted degradation. This is the first demonstration of a novel, simple, and generally applicable affinity cleavage strategy: use of catalytic antagonists. A low-molecular-weight targeting ligand (antagonist) locks the active site region of a proteinase (catalyst of destruction) in close proximity to the specific recognition site of a target protein. This focuses the degradative activity of the proteinase on a juxtaposed target that is not its preferred substrate. Correct positioning of the targeting ligand on the proteinase is critical. Indiscriminate positioning fails.^[7] In all examples studied so far, attachment of the targeting ligand at position 156 gave the best results. This engineered enzymatic degradation of a preselected protein target has been achieved with more than 350-fold enhanced selectivity compared to untargeted degradation.

The mode of action of CAs is catalytic^[29] (Figure 1) since once the ligand-binding site of the target protein has been sufficiently degraded, the CA becomes free to seek-and-destroy additional targets. The method utilizes easily prepared reagents and is potentially unlimited in the scope of degradative enzymes or targeting ligands that could be conjugated. The ease with which any CA can be redirected at will towards a selected target by simply retooling the enzyme with an alternative appropriate targeting ligand provides a variety of therapeutic opportunities. The advantages of CAs, such as enhanced activity and improved target discrimination, allowed the targeted destruction of

proteins from three important classes: binding proteins, immunoglobulins, and enzymes.

Design of therapeutic CAs would, of course, necessitate the selection of the most pharmaceutically effective, nonallergenic hydrolytic enzyme possible but such therapeutics could be administered at substoichiometric levels at potentially lower dosages than normally required for a similar drug. Our results are highly encouraging and represent steps towards therapeutically viable selectivity; further enhancement is an important goal.

We are grateful to Drs Douglas Crabb, Anthony Day, Andrew Shaw, Roopa Ghirnikar, and Cynthia Edwards for their critical reading of this manuscript and to T. Percy Hughes for useful discussions. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (JBJ) and Genencor International Inc. (JBJ and BGD), and by a scholarship from the Deutsche Forschungs Gemeinschaft (Germany; AU).

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 [26] Experiments were conducted by using a mixed substrate solution of target enzyme HLADH and decoy enzyme (CA or BG) in the same experiment to give a true measure of competitive selectivity for target over decoy.
 [27] The fragility of the decoy clearly influences the level of collateral damage; while the very robust protein bovine carbonic anhydrase is virtually undegraded, more fragile proteins will obviously require greater selectivity. Bovine β -galactosidase was chosen as an additional more fragile decoy than bovine carbonic anhydrase to allow a measure of selectivity. We are currently examining a range of decoys of differing fragility; clearly selectivity will also be decoy dependent.
 [28] Reduced degradation of BG is more apparent at longer reaction times and, in addition to being due to targeting-enhanced selectivity for HLADH, may also be the result of inhibition by low-molecular-weight peptides formed by protein degradation. The inhibitory effect of such protein fragment peptides is under investigation.
 [29] During this study the following ratios of CA to target were employed: CA/avidin, 1:13; CA/con-A, 1:20; CA/antibiotin-IgG, 1:2; CA/HLADH, 1:4.

Received: November 11, 2002 [Z591]

Binding and Catalysis: A Thermodynamic Study on a Catalytic Antibody System

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KEYWORDS:

calorimetry · catalytic antibodies · ester hydrolysis · phosphate · transition states

The idea that enzymes catalyze chemical reactions by binding transition states tightly has provided an important basis for generating molecules with catalytic activity.^[1a] The diverse

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