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On the rational design of substrate mimetics: the function of docking approaches for the prediction of protease specificities

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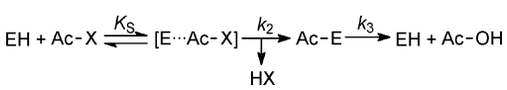
The behaviour of substrate mimetics in mediating the acceptance of nonspecific acyl moieties by proteases has been investigated as a direct function of their site-specific ester leaving groups. In this contribution we report on a computational approach to rationalise this interplay and to predict the power of a potential ester moiety to act as a suitable substrate mimetic for a given enzyme by means of an automated docking procedure. Investigations with seven distinct substrate mimetics and two proteases, subtilisin and chymotrypsin, show a clear correlation between the theoretically calculated binding energies ΔE and the specificity constants $k_{cat}\,K_M^{-1}$ obtained from parallel hydrolysis kinetic studies. These results prove the general function of the docking approach as a rational model not only in predicting the general acceptance of a substrate mimetic in a qualitative manner, but also to provide reliable information on its individual specificity towards proteases.

Introduction

Enzymes exhibit unique specificities and selectivities compared to traditional means of chemical catalysis making them attractive technological tools in applied chemical processes.[1] Especially for modifications of complex molecules with multiple stereocenters or a wide variety of functional groups they open up a new dimension of reaction control that cannot be achieved by classical chemical approaches. With these advantages, however, comes the disadvantage that the pronounced specificities reduce simultaneously the universality of enzymes as synthetic catalysts. Efforts to broaden the limited substrate spectrum of native enzymes are, therefore, of decisive importance for raising the scope of these catalysts for synthesis. Related to proteases, substantial improvements in the enzyme's synthesis flexibility have been attained by the development of substrate mimetics used as the acyl donor components.[1] Based on their unique architecture, which is typically characterized by a shift of the site-specific amino acid moiety from the *C*-terminus of the peptide residue to the substrate's ester leaving group, substrate mimetics mediate the acceptance of completely nonspecific acyl moieties. This behaviour enables the synthesis of a broad spectrum of compounds including for instance peptides,[2] isopeptides,[3] all-D-peptides,[4] peptide isosteres,[5] peptidoglycans[6] or nonpeptidic carboxylic acid amides.[7] Moreover, a function of the approach to the resolution of racemates has recently been proposed.[8] Furthermore, it has been shown that suitably adapted substrate mimetics are well accepted by virtually all serine and cysteine proteases independent of their individual native substrate preferences making the approach a rather general one in protease catalysis.[1] As suggested by attempts to visualize the structure of the substrate mimetic–enzyme complex formed during reaction, a specific binding of the ester leaving group to the specificity-determining S_1 position of the respective protease (nomenclature according to ref. 9) can be considered as the key event for mediating the acceptance of substrate mimetics.[10] Moreover, it became evident that for productive binding a conformation of the scissile ester bond similar to that of normal-type substrates must be arranged. Approaches to the screening of potential substrate mimetics used so far, however, are based on simple empirical comparisons of the structural similarity between the side chain of preferred amino acid residues and respective ester leaving groups and allow at best only very rough estimations of the general suitability of a given ester moiety.[11,12]

This contribution reports on the utility of an automated docking method to predict the acceptance and specificity of potential substrate mimetics to proteases of known 3D-structure. The general function of the approach benefits from the kinetics of substrate mimetic-mediated reactions that involve a fast acylation of the enzyme followed by a deacylation being rate limiting.[13] In cases where identical acyl moieties within the substrate mimetics are used, the rate of the latter can, therefore, be considered equal for the corresponding enzyme without having any influence on the specificity of the respective substrate (*cf*. Scheme 1). Consequently, the specificity of the individual substrate mimetic is exclusively encoded in the binding and acylation behaviour of the ester leaving group. The former can be calculated directly by the docking approach in terms of the binding energies while for estimating the efficiency of the acylation step the productivity of the computed substrate–enzyme complexes is analyzed.



Scheme 1 Kinetic model of protease-mediated substrate ester hydrolysis. EH, free enzyme; Ac-X, substrate ester; [E...Ac-X], Michaelis–Menten complex; HX, leaving group; Ac-E, acyl enzyme intermediate; Ac-OH, hydrolysis product.

Results and discussion

Theoretical calculations

We initially selected a set of seven individual types of substrate mimetics with respect to the primary specificity of subtilisin

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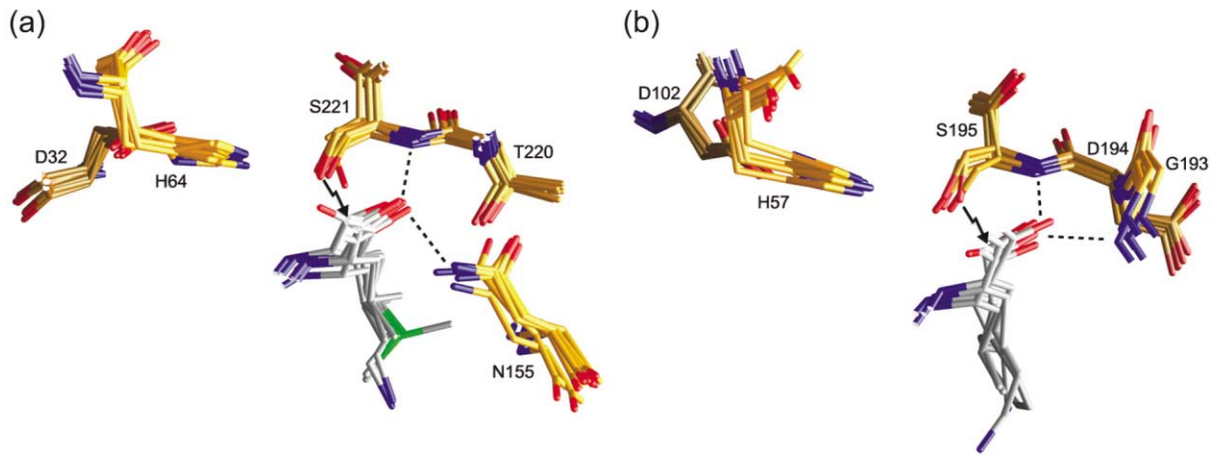
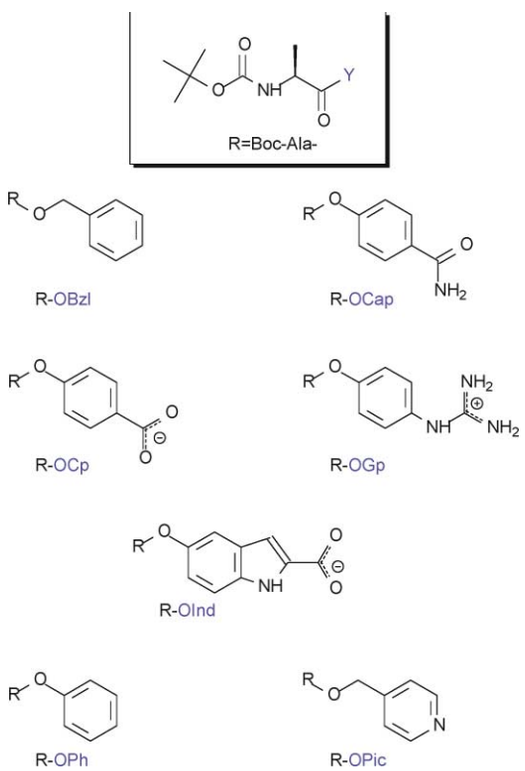


Fig. 1 Protease–ligand complexes of (a) subtilisin (pdb-entries:[14] 1av7, 1avt, 1cse, 1lw6, 1scn, 2sec, 3sec, 1sib, 2sic, 5sic, 2sni, 1sua and 3vsb) and (b) chymotrypsin (pdb-entries: 1acb, 1cho, 2cha, 6cha, 1hja and 1mtn). Only the residues of the catalytic triad, the residues forming the oxanion hole (orange) and the P_1-residue of the ligand (grey) are shown. The hydrogen bonds are drawn as dotted lines; the arrow indicates the attack of the catalytic serine.

and chymotrypsin. Since both proteases are well known to prefer originally aromatic amino acid moieties,[15] leaving groups derived from benzyl, indoyl and in particular phenyl esters have been empirically chosen as potential candidates. The latter were favoured because of the already reported acceptance of phenyl (OPh) and 4-guanidinophenyl (OGp) esters by chymotrypsin.[16] For selecting a suitable substitution pattern of the aromatic ring systems, earlier findings were considered which reveal a role of hydrogen bonds for stabilising the complex of the two proteases with their native substrates.[15] Thus, functionalities were preferred that act either as a donor or acceptor of hydrogen bonds. As the acyl residue, the *tert*-butyloxycarbonyl-alanine moiety has been chosen in all cases, which can be considered as a neutral and only very poorly accepted amino acid derivative by both proteases. A complete list of the ester structures selected is illustrated in Scheme 2.



Scheme 2 Structures of substrate mimetics: OBzl: benzyl ester; OCap: 4-amido carboxy phenyl ester; OCp: 4-carboxy phenyl ester; OGp: 4-guanidino phenyl ester; OInd: 2-carboxy-1*H*-indol-5-yl ester; OPh: phenyl ester; OPic: 4-picoyl ester.

Prior to synthesis, the esters were docked against the crystal structures of subtilisin and chymotrypsin, respectively, employing the program package AutoDock.[17] The calculated substrate–enzyme complexes showing the lowest binding energies were subsequently analysed for a catalytically productive orientation of the substrate mimetic within the active site of the respective protease. The criteria for a productive substrate mimetic–enzyme complex were defined after analysing 3D structures of subtilisin and chymotrypsin complexed with specific ligands. As illustrated in Fig. 1, all these protein–ligand arrangements share characteristic features of the orientation of the P_1 residue of the complexed compound with respect to the active site of the corresponding protease; *i.e.* the distance between the substrate's carbonyl carbon atom of the ester bond and the γ-oxygen atom of the active serine is less than 3 Å. Furthermore, the carbonyl oxygen of all ligands points towards the oxanion hole, which is formed by the backbone amide moiety of serine 221 and the side chain amide of asparagine 155 in the case of subtilisin and by the backbone amide groups of serine 195 and glycine 193 in the case of chymotrypsin. For detailed analysis of binding energies, only those substrate mimetic–protease arrangements fulfilling the aforementioned productive orientation have been considered. In most cases these were the most energetically favourable complexes or the highest populated complexes in the docking runs, respectively (*cf.* Experimental section).

Fig. 2 shows a snapshot of the individual binding of the substrate mimetics to the two proteases in their productive and energetically most preferred conformations. Importantly, all the complexes have in common that the ester leaving groups point into the S_1 binding pocket while the acyl moiety is located in the S′ region of the respective protease. In accordance to earlier studies,[10] such an arrangement can be considered typically for substrate mimetics which already indicates an acceptance of all substrate esters by the two enzymes.

A more rational and detailed picture becomes apparent from Table 1 listing the individual binding energies ΔE calculated for the productively bound substrate mimetics. Considering an intrinsic error of the energy evaluation method of approximately 1.5 kcal mol^{-1}, the ΔE values obtained for subtilisin are almost equivalent without displaying any significant deviations. This finding let one expect that the variation of the ester leaving group's structure should have almost no effect on the specificity of subtilisin towards the substrate mimetics involved in this study. This is in good agreement with the well-known broad specificity of subtilisin. On the contrary, a completely different situation is found for chymotrypsin. For this enzyme, the binding energies vary between −6.5 and −10.2 kcal mol^{-1} indicating significant differences in the specificity of chymotrypsin

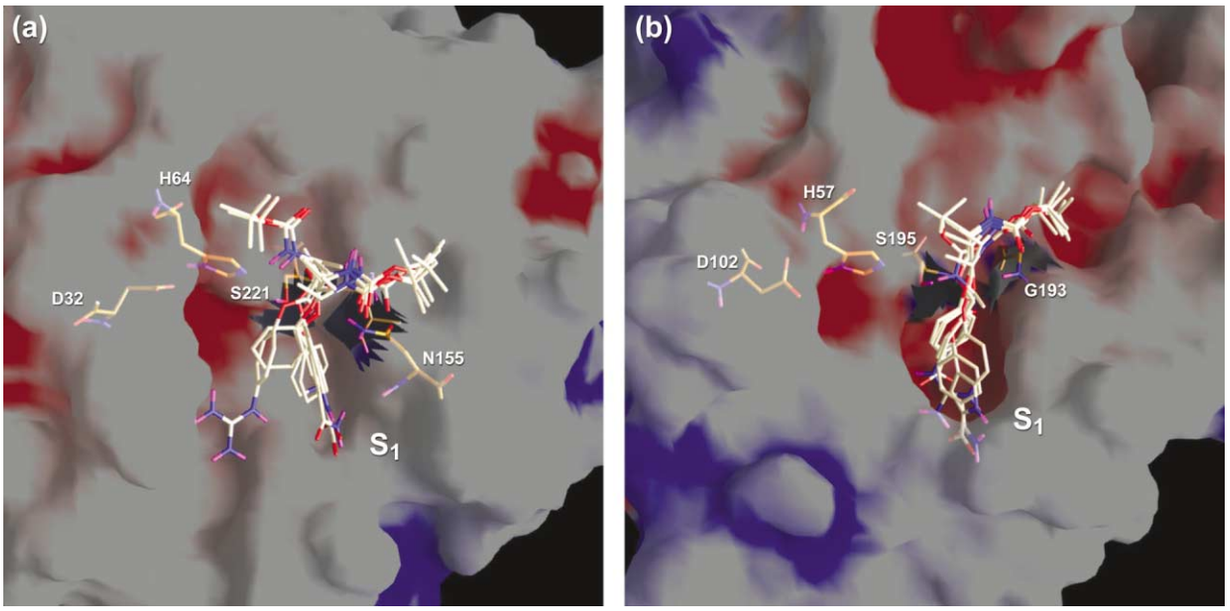


Fig. 2 Productive complexes of the investigated substrate mimetics docked to (a) subtilisin and (b) chymotrypsin. Surfaces of the proteins are coloured according to their electrostatic potential (blue = positive, red = negative) calculated with Grasp.[19] The residues of the catalytic triad and the residues of the oxanion hole are in orange.

Table 1 Binding energies of productive substrate–protease complexes calculated with AutoDock

	ΔE/kcal mol^{-1} [a]	
Ester	Subtilisin	Chymotrypsin
Boc-Ala-OBzl	−6.3	−7.9
Boc-Ala-OCap	−5.9	−8.9
Boc-Ala-OCp	−6.8	−6.5
Boc-Ala-OGp	−6.2	−9.2
Boc-Ala-OInd	−7.3	−10.2
Boc-Ala-OPh	−5.3	−7.1
Boc-Ala-OPic	−5.9	−7.7

[a] All calculated data have an intrinsic error of approximately 1.5 kcal mol^{-1}.[17]

Table 2 Specificity constants for the hydrolysis of substrate mimetics by subtilisin and chymotrypsin[a]

	$k_{cat}\ K_M^{-1}$/M^{-1} s^{-1} [b]	
Ester	Subtilisin	Chymotrypsin
Boc-Ala-OBzl	1.7×10^4	1.1×10^2
Boc-Ala-OCap	1.1×10^4	5.1×10^3
Boc-Ala-OCp	6.5×10^3	3.7×10^1
Boc-Ala-OGp	7.2×10^3	3.6×10^3
Boc-Ala-OInd	2.1×10^3	1.0×10^2
Boc-Ala-OPh	1.6×10^4	1.9×10^2
Boc-Ala-OPic	1.2×10^4	6.8×10^2

[a] Conditions: 0.1 M Hepes, pH 8.0, 0.1 M NaCl, 10 mM $CaCl_2$, 8% (v/v) DMF, 25 °C. [b] Errors are less than 15%.

towards the individual substrate esters with the lowest for Boc-Ala-OCp and the highest for Boc-Ala-OInd. Boc-Ala-OGp, which is already commonly used as a substrate mimetic for this enzyme,[16,18] shows a binding energy very close to that of Boc-Ala-OInd. Interestingly, a comparable value of ΔE was also calculated for Boc-Ala-OCap, which only differs from the worst ester Boc-Ala-OCp by having a neutral amide group instead of a negatively charged carboxylate at position 4 of the aromatic leaving group. Higher binding energies and, thus lower specificities were predicted for Boc-Ala-OBzl, Boc-Ala-OPic, and Boc-Ala-OPh, which themselves show ΔE values very close to each other.

Enzymatic hydrolysis

To prove the predictions made by the automated docking approach, the specificity constants have been experimentally determined by usual steady-state hydrolysis studies. Plotting of the initial rates of hydrolysis *vs.* the substrate ester concentration resulted in straight lines for all enzyme/substrate combinations (plots are not shown). From the slopes of the respective curves, the apparent second order rate constants $k_{cat}\ K_M^{-1}$ have been calculated (Table 2). Analysis of the resulting data show that all substrate esters were found to be specifically hydrolysed by the two proteases as it was predicted by the docking approach. Also in good agreement to the docking calculations are the similar $k_{cat}\ K_M^{-1}$ values of the distinct esters in the case of subtilisin, which only differ from each other by less than a factor of ten. Although less specific in total, chymotrypsin shows a more pronounced specificity profile towards the individual substrates with specificity constants varying by more than two orders of magnitude. Plotting of the specificity constants $k_{cat}\ K_M^{-1}$ *vs.* the calculated binding energies ΔE for subtilisin (Fig. 3) and chymotrypsin (Fig. 4) reflects clearly the different behaviour of the two proteases. Furthermore, it illustrates the correlation of the two values and, thus allows for

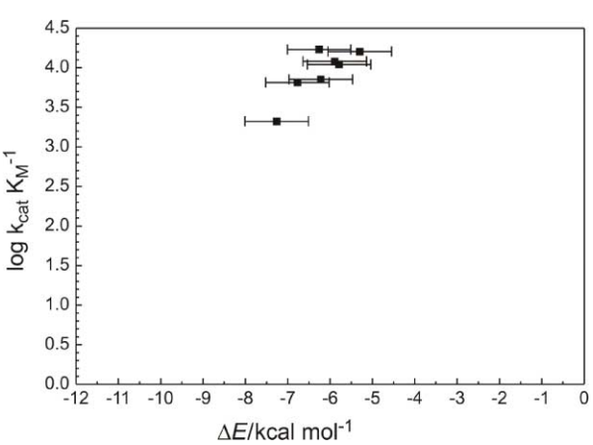


Fig. 3 Plot of specificity constants $k_{cat}\ K_M^{-1}$ of subtilisin-catalysed hydrolysis of substrate mimetics *vs.* calculated binding energies ΔE of the corresponding productive complexes.

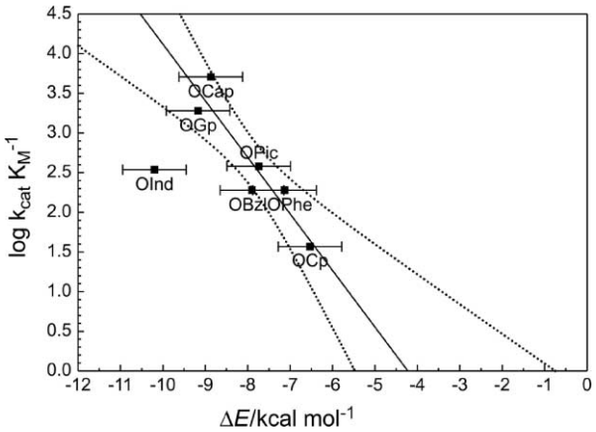


Fig. 4 Plot of specificity constants of chymotrypsin-catalysed hydrolysis of substrate mimetics *vs.* calculated binding energies of the corresponding productive complexes. The correlation coefficient is $R = 0.93$; the confidence interval of 95% is plotted as dotted lines. Correlation does not include Boc-Ala-OInd (*cf.* text).

conclusions as to the suitability of the docking approach for predicting the specificity of a given substrate mimetic in a quantitative manner. Analysis of Fig. 3 shows that the resulting data points in the case of subtilisin are almost located within the same area. Based on similar ΔE values, which would ideally correlate with similar values for $k_{cat}\ K_M^{-1}$, this finding matches exactly the prediction made by the docking approach for this enzyme. In general, a good correlation between the two values is also found for chymotrypsin (*cf.* Fig. 4). In this case, an increase in the binding energies comes along with an increase in the specificity constants. Linear regression of the data results in a correlation coefficient R of 0.93 with almost all the data located within a confidence interval of 95%. An exception was found, however, for Boc-Ala-OInd whose specificity constant is slightly lower than expected from the theoretical studies. As already pointed out, the applied computational docking approach relies on the calculation of binding energies and, thus, provides primarily information on the affinity of ligand binding. Assuming that the binding energy is calculated correctly and that the rate of deacylation is equal for all esters due to their identical acyl residues, this discrepancy should be caused by differences in the rate of acylation as the third remaining step of the reaction. Following this hypothesis, the lower specificity of chymotrypsin for hydrolysing Boc-Ala-OInd could be therefore the result of a reduced rate of acylation compared to the other ester types. Although this was apparently not the case for subtilisin, the reason for this different acylation behaviour might be based on the unique chemical structure of the indoyl ester. Among the substrate mimetics used, Boc-Ala-OInd is a bicyclic ring system while all other esters are derived from monocyclic phenyl or benzyl moieties. Due to the high electron density of the heterocyclic indoyl moiety a stabilising effect on the scissile ester bond can be expected which would lower the degree of ester bond activation and make the ester hydrolysis more demanding. The exclusion of Boc-Ala-OInd from the linear regression of the ΔE and $k_{cat}\ K_M^{-1}$ values results, however, in the aforementioned good correlation coefficient whereas Boc-Ala-OCap shows the highest binding energy and specificity constant as well.

Conclusions

The results of our studies show that automated docking methods based on the calculation of binding energies can be successfully applied to predict the general acceptance of a given substrate mimetic by proteases. Besides this qualitative prediction, the approach further allows for a quantitative estimation of the individual specificity of a potential substrate mimetic. Independent of the enzyme and substrate mimetic used, a good correlation between the calculated binding energies and the experimentally determined specificity constants has been found in almost all cases. Provided that the three-dimensional structure of a suitable protease is available, this computational method therefore enables a rational screening of de-novo designed substrate mimetics prior to their synthesis, which finally facilitates the identification of novel, and highly specific substrate mimetics significantly. Already within this initial study, Boc-Ala-OCap has been identified as an efficient and even slightly higher specific substrate mimetic for chymotrypsin than the previously used OGp ester. This is a result that clearly proves the potential of this approach. Broader studies with larger substrate mimetic libraries might lead to further promising candidates with even higher specificities. Studies in this direction including also a larger number of proteases are presently under investigation. Further efforts, however, are necessary to optimize the docking calculations in particular their capability to consider the degree of ester bond activation. Differences in the latter might be responsible for the non-correct prediction of the specificity of chymotrypsin towards Boc-Ala-OInd. At the present stage of development, best results can be expected for ester moieties of similar basic structures varying mainly in the substitution pattern.

Experimental

General

Boc-Ala-OH, coupling reagents, ester components, enzymes and buffer components were products of Bachem, Sigma, Fluka (Switzerland) or Merck (Germany). All reagents were of the highest purity commercially available. Solvents were purified and dried by the usual methods.

Molecular modelling and automated docking

The protein ligand docking studies were performed on the basis of the crystal structures of α-chymotrypsin complexed with phenylethane boronic acid (pdb entry 6cha[20]) and subtilisin (pdb-entry 1svn[21]). All solvent molecules, ions and the cocrystalised inhibitor were removed from the structures. Polar hydrogen atoms were added using the modelling package Quanta98 (Accelrys Inc., San Diego CA, 1998). After assignment of template charges the protein structures were subjected to a short geometry relaxation (500 steps steepest descent, CHARMm23 force field[22]). The various substrate esters were modelled in the same way, but with charges derived from their electrostatic potential, which were obtained from single point calculations at the AM1 level[23] of MO theory using the program Spartan 5.0 (Wavefunction Inc., Irvine CA, 1998).

For the docking calculations, the program package AutoDock 3.0 was employed. To allow the ligand to search the entire active site, a cubic box of 30 × 30 × 30 Å was centred on the γ-oxygen atom of the catalytic active serine (serine 195 for chymotrypsin and serine 221 for subtilisin). The search for the possible ligand binding sites was performed within this box at the default grid point spacing of 0.375 Å. All relevant torsion angles in the ester ligands were released. Each single docking experiment consisted of 30 runs employing a Lamarckian genetic algorithm and took a computing time of about 15 min on a standard Pentium-4 system to be finished. The docking runs were initiated with 50 randomly chosen protein–ligand arrangements and iterated through 5×10^6 energy evaluations. Up to 50 iterations of local search were applied with a frequency of 0.1. Thus, approximately 150 million protein–ligand arrangements were evaluated per substrate and enzyme. All other docking parameters were set to their default values. To ensure correct calculation of the free binding energy, aromatic carbon atoms were treated with an additional solvation term, as it is incorporated in the AutoDock program. The resulting protease–substrate complexes were clustered employing an

rmsd tolerance of 1 Å. Only clusters representing productive ligand–protein complexes were further analysed, which were in most cases the highest populated cluster or the cluster with the lowest binding energy, respectively.

Chemical syntheses

Boc-Ala-OGp was prepared according to our previously described protocols.[10] Similarly, Boc-Ala-OPh and Boc-Ala-OPic were synthesized by DCC coupling of the appropriate Boc-protected amino acid with phenol and 4-hydroxymethyl pyridine, respectively. Boc-Ala-OCap and Boc-Ala-OCp were prepared by coupling the ester bond using TBTU. The final amino acid esters were purified by preparative HPLC. The identity and purity of all final products were checked by analytical HPLC at 220 nm, NMR, thermospray mass spectroscopy, and elemental analysis (analytical data see ref. 24). In all cases, satisfactory analytical data were found (± 0.4% for C, H, N).

Enzyme kinetics

Hydrolysis reactions were performed in a total volume of 450 µl at 25 °C. Stock solutions of Boc-Ala-OY esters were prepared in 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, and 10 mM $CaCl_2$ containing 8% (v/v) DMF as cosolvent. The substrate concentrations were between 0.1 and 9.0 mM and the enzyme concentrations between 6.4×10^{-8} and 3.2×10^{-6} M. The active enzyme concentration was determined by active-site titration using 4-nitrophenyl acetate.[25] After thermal equilibration of assay mixtures, the reactions were initiated by addition of the appropriate enzyme stock solutions. Subsequently, the mixtures were rapidly shaken and transferred into a thermomixer adjusted to 25 °C. The reaction rates were analyzed by RP-HPLC determining the disappearance of the substrate esters by at least ten different concentrations. For this purpose, 50 µl aliquots were withdrawn at defined time intervals and diluted with a quenching solution of 50% methanol containing 5% TFA. As a control for the spontaneous hydrolysis of the acyl donor esters, parallel reactions without enzyme were analyzed. On the basis of these control reactions the extent of spontaneous hydrolysis was found to be less than 5%. The kinetic parameters were calculated by iterative nonlinear curve fitting of the untransformed data using the program SigmaPlot Scientific Graphic System (Version 6.10, SPSS Inc.). The data reported are the average of at least three independent experiments.

HPLC analyses

HPLC measurements were performed with a Shimadzu LC-10A HPLC system using a LiChrospher RP 18 column (250 mm × 4 mm, 5 µm, Merck, Germany) or a Capcell PAK C8 column (250 mm × 4 mm, 5 µm, Shiseido, Japan). Samples were eluted with various mixtures of water and acetonitrile containing 0.1% TFA under isocratic and gradient conditions at flow rates of 1.0 ml min^{-1}. Detection was at 254 nm. The reaction rates were calculated from peak areas of the ester substrates using 4-toluenesulfonic acid as an internal standard.

Acknowledgements

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