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PROBING THE SPECIFICITY OF THE S_1 BINDING SITE OF M222 MUTANTS OF SUBTILISIN *B. LENTUS* WITH BORONIC ACID INHIBITORS

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Abstract: Specificity differences between the S_1-pockets of subtilisin *B. lentus* (SBL), and its M222C/S mutants have been explored with boronic acid inhibitors. Similar binding trends were noted, with 2,4-dichlorophenylboronic acid being the best overall inhibitor for each enzyme. In addition, a correlation between inhibitor binding and the electrophilicity of boron was found for both the M222C and M222S enzymes.

Enzymes are now widely accepted as useful catalysts in organic synthesis.[1] Furthermore, interest in enzyme-catalyzed reactions has been intensified, particularly in industry, by the current focus on developing environmentally benign synthetic methods. Since enzymes operate best in the nontoxic and nonpolluting solvent, water, they are particularly suited for industrial applications. However, despite the extensive synthetic applications of enzymes, relatively little is known about the factors that control their structural- and stereospecificities towards unnatural substrates and inhibitors, nor of the strategies needed to apply site-directed mutagenesis to tailor their specificities at will. It is the need to expand the database on these aspects that stimulated the current study.

Boronic acids have been shown to be effective reversible transition state inhibitors of serine proteases,[2] whose value as competitive inhibitor probes of subtilisin Carlsberg (SC) specificity has been established.[3] Accordingly, as the first steps towards clarifying the factors involved in controlling and modifying the specificities of subtilisin *Bacillus lentus* (SBL) and its mutants, we have examined the influence of M222C and M222S (BPN′ numbering) mutations on the binding of boronic acid inhibitors to the hydrophobic S_1 -pocket. Position 222, while formally part of the S_1' pocket, is adjacent to S_1 and also to the active serine, 221, of the catalytic triad. Site-directed mutagenesis of the WT-M222 residue is expected to influence both the S_1 subsite and catalytic triad environments and properties. In this paper, some effects of M222S and M222C mutations are reported.

Results and Discussion

The gene for subtilisin from *B. lentus* was cloned into the bacteriophage M13mp19 vector for mutagenesis.[4] Oligonucleotide-directed mutagenesis was performed as described previously.[5] The mutated sequences were cloned, excised, and reintroduced into the expression plasmid GGT274 in the *B. subtilis* host. The crude protein concentrate containing PEG (50%) as a stabilizer was purified by passing it through a Sephadex G-25 desalting matrix with a pH 5.2 buffer (20 mM sodium acetate, 5 mM $CaCl_2$), to remove small molecular weight contaminants. Pooled fractions from the desalting column were then applied to a strong cation exchange column (SP Sepharose FF) in the same buffer, and SBL was eluted with a one-step gradient of 0-200 mM NaCl-acetate buffer, pH 5.2. Salt-free enzyme powder was obtained by dialysis of the eluent against Millipore-purified water, and subsequent lyophilization. The purity of the mutant and WT enzymes, denatured by incubation with 0.1 M HCl at 0 °C for 30 min, was ascertained by SDS-PAGE on 20% homogeneous gels using the Phast System from Pharmacia. The concentration of SBL was determined using the BioRad dye reagent kit, which is based on the method of Bradford.[6] Specific activity of the enzymes was determined in pH 8.6 buffer (0.1 M TRIS, 0.005% Tween 80, 1% DMSO) using Suc-AAPF-pNA (Sigma, 1 mg/mL) as the substrate.

Table 1. Kinetic Parameters for Subtilisin *B. lentus* Enzymes[a]

Enzyme (structure at 222)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
 (WT)	48 ± 2	0.55 ± 0.06	8.7×10^4
 (M222C)	17.3 ± 0.4	0.77 ± 0.05	2.2×10^4
 (M222S)	29.5 ± 6	1.03 ± 0.27	2.9×10^4

(a) Michaelis-Menten constants were measured by the initial rates method in 0.1 M phosphate buffer containing 0.5 M NaCl at pH 7.5 with Suc-AAPF-pNA as the substrate. Active enzyme concentrations were determined via PMSF titrations.[7]

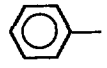
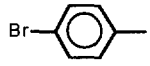
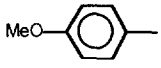
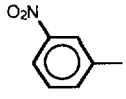
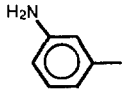
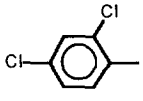
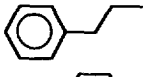
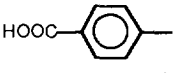
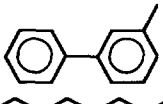
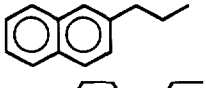
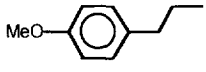
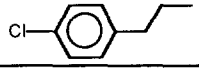
The kinetic constants for SBL-WT along with its M222S and M222C mutants were determined by the method of initial rates in 0.1 M phosphate (pH 7.5) buffer containing 0.5 M NaCl, with Suc-AAPF-pNA as the substrate. Kinetic runs were performed in duplicate and the data processed using the Enzyme Fit program in GraFit®. The results are recorded in Table 1.

Since Met222 is a generally conserved residue among subtilisins, it is interesting to note that the mutation of SBL's methionine at this position to the more hydrophilic and polarizable cysteine or serine residues results in a decrease in k_{cat} and an increase in K_M. The 3- to 4-fold decrease in k_{cat}/K_M for both mutants is attributed to a decrease in the hydrophobicity of the environment adjacent to the S_1 pocket and the catalytic serine 221 residue.

We then turned our attention to evaluating the effects of 222 mutations on the S_1 - pocket properties of SBL, using the same screen of boronic acid inhibitors employed in our previous specificity probing study of SC.[3] The boronic acids were prepared and their inhibition constants measured,[8] as described previously.[3] The results are recorded in Table 2. While the inhibition by compounds **1**, **2**, **4**, **6**, and **10** remained relatively constant for SBL-WT, M222C, M222S, and SC, widely varying K_I values were observed for inhibitors **3**, **5**, **7**, **9**, **11**, and **12**. The best overall inhibitor in the series, 2,4-dichlorophenylboronic acid (**6**), displays a K_I that is essentially constant for the four enzymes. However, the most interesting results are for 3-biphenylboronic acid (**9**), where the M222C and M222S mutants show 10- and 5-fold better binding, respectively, than for SBL-WT. Because both the serine and cysteine side chains are smaller than that of the wild-type methionine, the larger boronic acids are thus able to penetrate into the S_1 pocket of M222S and M222C with greater facility than for the more sterically hindered wild-type enzyme. Such steric hindrance, also apparent with the poorer binding of 3-biphenylboronic acid (**9**) and naphthylethylboronic acid (**10**) to SBL-WT relative to SC, is consistent with the smaller binding pocket of SBL.[9]

It is particularly interesting that the inhibition constants for phenethylboronic acid (**7**) and *para*-chlorophenethylboronic acid (**12**) do not parallel the results previously documented for SC.[3] The 19-fold better binding of the *para*-chlorophenethyl inhibitor (**12**) relative to phenethylboronic acid (**7**) with SC was previously ascribed to the favorable interaction of the highly electronegative chlorine substituent with an electropositive region at the bottom of SC's S_1 pocket.[3] Minimizations of the EI complexes of each of these inhibitors, as their covalently linked complexes to the serine 221 hydroxyl of SBL-WT, were performed using the BioSym Discover Program in order to provide further insights into the binding modes of the boronic acids. In each case the aromatic portion of each inhibitor positioned itself unequivocally in the hydrophobic S_1 pocket. In addition, the BioSym Delphi program was employed to determine whether or not electrostatic attractions also contributed to binding, as had been found to be the case for SC.[3] However, the clearly positive region at the bottom of S_1 for SC was not apparent for SBL, and the molecular modelling data provided no evidence for any

Table 2. K_I - Constants for Competitive Inhibition by Boronic Acids

Inhibitor $RB(OH)_2$	R	K_I (μM) SBL-WT[a]	M222S[a]	M222C[a]	SC[b]
1		141 ± 22	96 ± 39	155 ± 36	100 ± 3
2		23 ± 4	10 ± 4	23 ± 5	23 ± 1
3		577 ± 78	310 ± 127	757 ± 174	180 ± 10
4		27 ± 4	15 ± 6	38 ± 8	8 ± 0.7
5		150 ± 23	73 ± 30	204 ± 47	75 ± 5
6		9.2 ± 1.4	8 ± 3	18 ± 4	10 ± 0.8
7		801 ± 68	95 ± 39	241 ± 5	257 ± 19
8		802 ± 108	2100 ± 910	847 ± 194	---
9		327 ± 50	69 ± 29	31 ± 7	37 ± 2
10		1584 ± 215	>1000	>1000	120 ± 8
11		986 ± 134	219 ± 91	314 ± 72	58 ± 4
12		325 ± 44	110 ± 45	233 ± 53	19 ± 1

(a) K_I values were determined in duplicate at 25 °C in 0.1 M phosphate buffer containing 0.5 M NaCl and 1% DMSO, pH 7.5, with Suc-AAPF-pNA as the substrate by the method of Waley.[8]
(b) From reference 3.

significant differences between the binding modes for the two inhibitors **7** and **12** in SBL, which is consistent with the ≤ 2-fold differences in K_I values observed experimentally.

Electron donating groups such as *p*-methoxyphenyl, *o*-aminophenyl, and *p*-methoxyphenethyl, as in **3**, **5**, and **11**, respectively, caused a decrease in the binding affinities of the inhibitors for each of the WT-SBL, M222S and M222C catalysts. In contrast, inhibitors with electron withdrawing substituents resulted in increased binding affinity, as illustrated for *p*-bromophenyl boronic acid (**2**) and *o*-nitrophenyl boronic acid (**4**).

In order to ascertain whether or not the variations in inhibitory powers of the phenylboronic acids in Table 2 were due to differences in the relative electrophilicities of the respective boron atoms, the K_I values for the inhibitors **1-5** and **8** were plotted against σ in a Hammett-type fashion, but no linear relationship was apparent. However, the latter analysis did not take desolvation factors into account. This was therefore done using the equation: $K_I = 1/K_{bind}K_{desolv}$ and calculating the values of $1/K_{bind}$, where $1/K_{bind} = K_I/e^{-\Delta Gdesolv/RT}$, according to the same procedure previously applied for SC.[3] Values of ΔG_{desolv} from the literature[10] were used. Using these desolvation-corrected data, Hammett-type plots of log K_{bind} vs. σ for SBL-WT, M222C, and M222S with compounds **1-4** exhibited positive ρ values of 4.34 ± 0.08, 4.28 ± 0.18 and 4.40 ± 0.23, respectively (Figure 1), consistent with a direct correlation between binding and the electrophilicity of boron, as was observed for SC.[3]

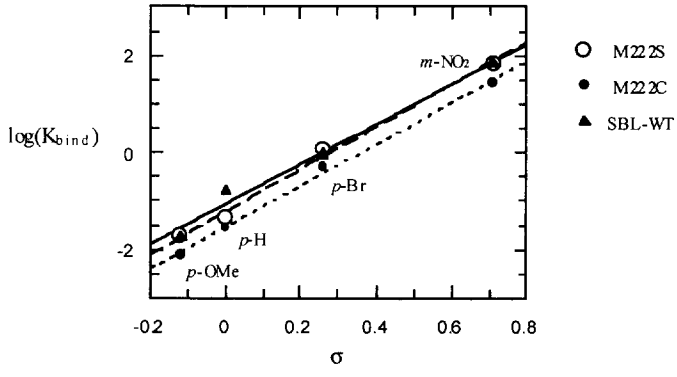


Figure 1. Hammett-plot correlations of the effects of substituents on the solvation corrected inhibition constant (K_{bind}) for phenylboronic acid inhibition of SBL-WT (▲ —), M222C (● ----) and M222S (O ---). Substituents are: *p*-OMe (**3**), *p*-H (**1**), *p*-Br (**2**), and *m*-NO_2 (**4**).

The suitability of the boronic acid screen as an effective probe of the S_1-pocket specificity is evident from the widely varying binding affinities for the WT, M222S, and M222C mutant enzymes, for which the K_I values vary from 8 μM to >1 mM, and from the binding affinity differences of up to 10-fold between the WT and mutant enzymes. The most notable differences between the SBL and SC series are the apparently greater steric constraints of the S_1 pocket, and the absence of a region of positive electrostatic potential at the base of S_1, in SBL.

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