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PROBING THE SPECIFICITY OF THE S_1 BINDING SITE OF SUBTILISIN CARLSBERG WITH BORONIC ACIDS

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Summary: The binding properties and limitations of the key S_1 site of subtilisin Carlsberg have been probed with boronic acid inhibitors bearing structurally varied substituents ranging from small alkyl to large aromatic groups. The data permit structural features favoring, and disfavoring, good S_1 binding to be clarified. In addition, applications of electrostatic energy calculations have identified a hitherto unsuspected region of positive potential in the fundamentally hydrophobic S_1 pocket, whose interactions with electronegative substituents of inhibitors can make significant binding contributions.

Despite the importance of hydrolytic enzymes in organic synthesis (1), the factors responsible for enzyme specificity towards unnatural substrates generally remain poorly understood. The contributions to binding specificity by factors such as hydrogen bonding, and hydrophobic and electrostatic interactions, are commonly probed by measuring the kinetic constants for substrate hydrolyses. These approaches can yield information about the relative importance of the different binding forces (2). Unfortunately however, such collections of kinetic parameters are often of limited use in identifying novel inhibitors in drug-design projects, and provide little insight to the organic chemist seeking suitable enzymes for asymmetric transformations of previously unknown substrates of synthetic intermediate value.

We became interested in this area because of the need to understand substrate and inhibitor binding factors as a prerequisite for optimizing and tailoring enzymes to cope with the broadest structural range of substrates in organic synthesis (3). As a further step in this direction, we recently initiated a program to analyze the binding of selected substrates and inhibitors to synthetically useful proteases, using a combination of enzyme kinetics and molecular modelling (4), in order to develop an understanding of the active site interactions involved that will be of predictive value for new, unnatural, substrate and inhibitor structures.

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Subtilisin Carlsberg (EC 3.4.21.14) was chosen as the initial model for these studies, since it is a commercially available serine protease that has been applied synthetically (5) and for which a high resolution X-ray crystal structure is available (6). A considerable amount of kinetic data has been published for this enzyme (3, 7), but the kinetically dictated substrate structures employed, and the wide range of assay conditions applied, render these data unsuitable for synthetically oriented molecular modelling analyses.

As the first step in this project, we have begun to map and explore the dimensions and binding features of the generally hydrophobic S_1 binding pocket (8) of subtilisin Carlsberg using boronic acid inhibitors of selected structural patterns. Boronic acids have been widely recognized as transition state inhibitors of serine proteases (9) and have recently been applied in active site mapping studies on such enzymes (10). Boronic acids act as competitive inhibitors that block the enzymic activity by forming tetrahedral adducts involving the catalytically active serine residue (11). Since such borate complexes are analogous to the tetrahedral intermediates involved in substrate hydrolysis (9, 12), boronic acids are well suited as substrate analogue probes of the binding specificity of serine proteases such as subtilisin Carlsberg. In this communication we report the partial dimensional mapping of the S_1 binding pocket of subtilisin Carlsberg using the boronic acid inhibitors **1-17**, and the molecular modelling identification of an electrostatically positive binding site at the bottom of the S_1 pocket.

Materials and Methods

Subtilisin Carlsberg, N-p-tosyl-L-arginine methyl ester (TAME) and butylboronic acid (**2**) were purchased from Sigma Chemical Company. Methylboronic acid (**1**), bromobenzene, 4-bromoanisol, (2-bromoethyl)benzene, 1-bromo-3-phenylpropane, 2-methylphenylacetic acid, 3-methylphenylacetic acid, 4-methylphenylacetic acid, 2-methoxyphenylacetic acid, 3-methoxyphenylacetic acid, 4-methoxyphenylacetic acid, 4-chlorophenylacetic acid, 1-naphthylacetic acid, 2-naphthylacetic acid and 2-cyclohexylethanol were purchased from Aldrich Chemical Company. 3,5-Dichlorophenylboronic acid was a gift from Prof. H. Hönig, Graz University of Technology, Austria.

Synthesis of Inhibitors: Boronic acids were prepared as described by Bean and Johnson (13). Bromo-starting materials that were not available commercially were prepared conveniently in two steps from the corresponding acids by reduction with $LiAlH_4$, followed by treatment with CBr_4. The resulting bromides were converted to the corresponding Grignard reagents and then reacted with trimethyl borate at low temperature to give the corresponding boronic acids. In our hands purification of the crude boronic acids proved to be difficult and they were reacted directly with ethylene glycol. The resulting ethylene glycol boronates were purified by distillation and then hydrolyzed to give pure samples of the corresponding boronic acids.

Kinetic Measurements: The initial rate of subtilisin Carlsberg-catalyzed hydrolysis of TAME was determined using a pH-Stat. All rates were determined at 25 °C on reaction mixtures containing 1 M KCl (1mL), 0.37 M aqueous TAME solution (0.4, 0.6, 0.8, 1.0, 2.0, 4.0 and 8.0 mL) and saturated aqueous inhibitor solution ($1x10^{-5}$-$1x10^{-2}$ M). In each case water was added to bring the final volume to 10 mL to give final concentrations of substrate in the range of 0.015 to 0.30 M and 10^{-6}-10^{-3} M of inhibitor. Boronic acids concentrations were titrated with 0.2 M NaOH solution to accurately

determine the boronic acid content. After equilibration for 5 minutes, the pH was adjusted to 7.8 with 0.2 M NaOH and the reaction initiated by addition of 50ul subtilisin stock solution (7.33×10^{-5} M in 0.1 M phosphate buffer pH 7.8). The uptake of 0.2 M NaOH was recorded directly into a PC and K_I's were determined using the Grafit scientific data analysis program (14).

Molecular Modelling: Calculations were performed on a Silicon Graphics 240GTX using the Delphi and Insight II programs from Biosym Technologies, La Jolla, California.

Results and Discussion

The inhibition constants (K_I) for the inhibition of subtilisin Carlsberg by the boronic acids **1-17** are recorded in Table I. Methylboronic acid (**1**), the smallest possible boronic acid, is a very poor inhibitor, having a K_I of 13mM. Nevertheless, this compound is an interesting inhibitor since it experiences minimal stabilization from S_1-pocket binding and therefore provides insight into the binding energy due to the boronic acid functional group alone. The incorporation of larger alkyl functions into the boronic acid, such as butyl (in **2**) and cyclohexyl (in **3**) does lower the K_I somewhat due to improved binding in S_1, but all three alkyl boronic acids have to be considered poor inhibitors.

In contrast, the boronic acids bearing aromatic groups are very good inhibitors. The three phenylboronic acids **4-6** are seen to be particularly effective. Since these compounds are too small to fit optimally into the S_1 binding site we interpret the tight binding that the low K_I's reflect to be due to the higher electrophilicity of the boron in

Table I. Inhibition of subtilisin Carlsberg with Boronic Acids

#	Boronic acid	K_I (mM)[a]
1	Methylboronic acid	13
2	Butylboronic acid	1.3
3	2-Cyclohexylethylboronic acid	3.3
4	Phenylboronic acid	0.067
5	4-Methoxyphenylboronic acid	0.18
6	3,5-Dichlorophenylboronic acid	0.003
7	2-Phenylethylboronic acid	0.27
8	3-Phenylpropylboronic acid	0.36
9	2-(2-Methylphenyl)ethylboronic acid	0.58
10	2-(3-Methylphenyl)ethylboronic acid	0.25
11	2-(4-Methylphenyl)ethylboronic acid	0.30
12	2-(2-Methoxyphenyl)ethylboronic acid	0.71
13	2-(3-Methoxyphenyl)ethylboronic acid	0.40
14	2-(4-Methoxyphenyl)ethylboronic acid	0.078
15	2-(4-Chlorophenyl)ethylboronic acid	0.037
16	2-(1-Naphthyl)ethylboronic acid	0.11
17	2-(2-Naphthyl)ethylboronic acid	0.12

[a] Inhibition of the subtilisin Carlsberg-catalyzed hydrolysis of N-p-tosyl-L-arginine methyl ester (K_M = 34 mM, k_{cat} = 65 s^{-1}).

the phenylboronic acids, which are stronger acids than the other Table I inhibitors in which aliphatic moieties are connected to the boron atom. For example, although the 2-phenylethyl function of **7** (K_I = 0.27 mM) binds more strongly into S_1 than the phenyl group of **4**, the latter is a much better inhibitor (K_I = 0.067 mM). This is a result of **4** (pK_a = 8.8 (15)) being a stronger acid than **7** (pK_a = 10.0 (15)). This explanation is also consistent with observations that electron withdrawing substituents such as chloro (as in **6**) enhance, and electron donating substituents such as methoxy (as in **5**) decrease, the efficiency of binding of phenylboronic acids to subtilisin Carlsberg.

2-Phenylethylboronic acid (**7**), with a K_I of 0.27 mM, is a powerful inhibitor of the hydrolysis of TAME. There is no significant change in the inhibition constant if the aromatic ring in **7** is substituted with methyl groups in either the meta- or para-positions, as in **10** or **11** respectively, or with a methoxy group in the meta-position, as in **13**. Chain extension by one CH_2, represented by 3-phenylpropylboronic acid (**8**), also has little effect on the inhibition constant. In contrast, the two ortho substituted inhibitors **9** and **12** exhibit enhanced K_I's, which is indicative of steric crowding in the upper part of the S_1 binding site.

In this context, it is interesting to note that 2-phenylethylboronic acid (**7**) exhibits a 10-fold lower K_I than 2-cyclohexylethylboronic acid (**3**). Preliminary modelling suggests that the reason for this is that the S_1 pocket is too narrow to accommodate the 6-membered ring of **3** in its conformationally preferred chair form. This results in a less than optimally oriented enzyme-boronic acid adduct. In contrast, the aromatic ring in **7** fits almost perfectly into the S_1 groove. Preferential binding of 2-phenylethyl- over n-alkylboronic acids has previously been reported for subtilisin Novo (16), for which the active site is similar in the S_1 region. (6)

The most interesting results were those obtained for 2-(4-methoxyphenyl)ethyl boronic acid (**14**) and 2-(4-chlorophenyl)ethyl boronic acid (**15**). These two compounds are up to 10-fold more effective inhibitors than their unsubstituted parent **7**. It was possible to explain these results by examining the patterns of electrostatic potential in the S_1 pocket of the enzyme. A Delphi calculation (17) of the active site region of subtilisin Carlsberg at pH 7.8 reveals a region of positive charge of between +0.2 to +0.4 located in the area of residues Gly 127 and Gly 128 near the bottom of S_1. Comparative calculations of the EI complexes with inhibitors **7** and **15** optimally docked, and with the para-chloro substituent of **15** in this positive S_1 region, predicted, that the binding of the para-chloro substituted inhibitor **15** should be favored by >1 kcal/mole relative to **7**. This is in agreement with the experimentally observed difference in inhibition constants, which shows that binding of **15** to subtilisin Carlsberg is stabilized by $\Delta\Delta G$ = 1.2 kcal/mole relative to **7**.

Finally, the behavior of the two naphthylboronic acids **16** and **17** was examined. These two boronic acids are excellent inhibitors, having K_I values half of that of 2-phenylethylboronic acid (**7**). The reasons for this improved binding of the naphthyl boronic acids is not yet clear, but evidently the limits of S_1's capacity for

accommodating large groups have not yet been reached. Additional boronic acid structures are now being made to probe further the limits and properties of the S_1 site of the enzyme.

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References

1. (a) Jones, J.B. (1986) Tetrahedron 42, 3351-3403. (b) Davies, H.G., Green, R.H., Kelly, D.R., and Roberts, S.M. (1990) Biotransformations in Preparative Organic Chemistry; Academic Press: London.
2. Philipp, M., and Bender, M.L. (1983) Molecular and Cellular Biochemistry 51, 5-32.
3. Bonneau, P.R., Graycar, T.P., Estell, D.A., and Jones, J.B. (1991) J. Am. Chem. Soc. 113,1026-1030 and references cited therein..
4. Karplus, M., and Petsko, G.A. (1990) Nature 347, 631-639.
5. (a) Delinck, D.L., and Margolin, A.L. (1990) Tetrahedron Lett. 31, 3093-3096. (b) Pugniere, M., San Juan, C., and Previero, A. (1990) Tetrahedron Lett. 31, 4883-4886. (c) Gotor, V., Garcia, M.J., and Rebolledo, F. (1990) Tetrahedron Asymmetry 1, 277-278. (d) Margolin, A.L., Delinck, D.L., and Whalon, M.R. (1990) J. Amer. Chem. Soc. 112, 2849-2854. (e) Chenevert, R., Desjardins, M., and Gagnon, R. (1990) Chem. Lett. 33-34. (f) Chenevert, R., Letourneau, M., and Thiboutot, S. (1990) Can. J. Chem., 960-963.
6. Mc Phalen, C.A., and James M.N.G. (1988) Biochemistry 27, 6582-6598.
7. (a) Wong, C.-H., Chen, S.-T., Hennen, W.J., Bibbs, J.A, Wang, Y.F., Liu, J.L.-C., Pantoliano, M.W., Whitlow, M., and Bryan, P.N., (1990) J. Amer. Chem. Soc. 112, 945-953. (b) Karasaki, Y., and Ohno, M, (1979) J. Biochem. 86, 536-537. (c) Karasaki, Y., and Ohno, M, (1978) J. Biochem. 84, 531-538. (d) Williams, A., and Woolford, G. (1972) J. Chem. Soc., Perkin Trans. II, 272-275.
8. (a) Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res Commun. 27, 157-162. (b) Berger, A., and Schechter, I. (1970) Philos. Trans. Roy. Soc. London, Ser. B 257, 249-264.
9. Demuth, H.-U. (1990) J. Enzym Inhibition 3, 249-278.
10. (a) Sutton, L.D., Lantz, J.L., Eibes, T., and Quinn, D.M. (1990) Biochim. Biophys. Acta 1041, 79-82. (b) Goz, B., Ganguli, C., Troconis,M., Wyrick, S., Ishaq, K.S., and Katzenellenbogen, J.A. (1986) Biochemical Pharmacology 35, 3587-3591. (c) Antonov, V.K., Ivanina, T.V., Berezin, I.V., and Martinek, K. (1970) FEBS Letters 7, 23-25.
11. (a) Matthews, D.A., Alden, R.A., Birktoft, J.J., Freer, S.T., and Kraut, J. (1975) J. Biol. Chem. 250, 7120-7126. (b) Tulinsky, A., and Blevins, R.A. (1987) J. Biol. Chem. 262, 7737-7743.
12. (a) Rawn, D., and Lienhard, G.E. (1974) Biochemistry 13, 3124-3130. (b) Bone, R., Shenvi, A.B., Kettner, C.A., and Agard, D.A. (1987) Biochemistry 26, 7609-7614. (c) Bone, R., Frank, D.,Kettner, C.A., and Agard, D.A. Biochemistry 28, 7600-7609.
13. Bean, F.R., and Johnson, J.R. (1932) J. Am. Chem. Soc. 54, 4415-4425.
14. Leatherbarrow, R.J. (1989) Grafit Erithacus Software Ltd., Staines, U.K.
15. Yabroff, D.L., Branch, G.E.K., and Bettman, B. (1934) J. Am. Chem. Soc. 56, 1850-1857.
16. Rotanova, T.V., Vasileva, T.V., Ginodman, L.M., and Antonov, V.K. (1978) Bioorg. Khim. 4, 694-698.
17. (a) Sharp, K., Fine, R., Gilson, M., and Honig, B. (1987) UCLA Symposium on Molecular and Cell Biology, Vol 69: Protein Structure, Folding and Design, Oxender, D.L. (Ed), 235. (b) Gilson, M., and Honig, B. (1987) Nature 330, 84-86.