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Benzophenone Boronic Acid Photoaffinity Labeling of Subtilisin CMMs to Probe Altered Specificity

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Abstract—A transition state analogue inhibitor, boronic acid benzophenone (BBP) photoprobe, was used to study the differences in the topology of the S_1 pocket of chemically modified mutant enzymes (CMMs). The BBP proved to be an effective competitive inhibitor and a revealing active site directed photoprobe of the CMMs of the serine protease subtilisin *Bacillus lentus* (SBL) which were chemically modified with the hydrophobic, negatively charged and positively charged moieties at the S_1 pocket S166C residue. As expected, in all cases BBP bound best to WT-SBL. BBP binding to S166C-$SCH_2C_6H_5$ and S166C-CH_2-*c*-C_6H_{11}, with their large hydrophobic side chains, was reduced by 86-fold and 9-fold, respectively, compared to WT. Relative to WT, BBP binding to the charged CMMs, S166C-S-$CH_2CH_2SO_3^-$ or S166C-S-$CH_2CH_2NH_3^+$, was reduced 170-fold and 4-fold respectively. Photolysis of the WT-SBL–BBP enzyme–inhibitor (EI) complex, inactivated the enzyme and effected the formation of a covalent crosslink between WT and BBP. The crosslink was identified at Gly127 by peptide mapping analysis and Edman sequencing. Gly127 is located in the S_1 hydrophobic pocket of SBL and its modification thus established binding of the benzophenone moiety in S_1. Photolysis of the EI complex of S166C-$SCH_2C_6H_5$, S166C-S-$CH_2CH_2SO_3^-$, or S166C-S-$CH_2CH_2NH_3^+$ and BBP under the same conditions did not inactivate these enzymes, nor effect the formation of a crosslink. These results corroborated the kinetic evidence that the active site topology of these CMMs is dramatically altered from that of WT. In contrast, while photolysis of the S166C-CH_2-*c*-C_6H_{11}–BBP EI complex only inactivated 50% of the enzyme after 12 h, it still effected the formation of a covalent crosslink between the CMM and BBP, again at Gly127. However, this photolytic reaction was less efficient than with WT, demonstrating that the S_1 pocket of S166C-CH_2-*c*-C_6H_{11} is significantly restricted compared to WT, but not as completely as for the other CMMs.

Introduction

Enzymes are widely exploited as catalysts in organic synthesis due to their regio-, stereo-, and enantio-selectivity under very mild reaction conditions.[1–3] In order to expand the synthetic utility of enzymes even further, additional insights into the precise nature of the factors which control their structural and stereospecificity are needed.[4,5] Consequently, the creation of enzymes with new catalytic activities, and tailoring the specificity of existing ones to accommodate unnatural substrates has become an area of current intense interest.[6]

Recently, the combined chemical modification–site-directed mutagenesis approach has emerged as an efficient method to tailor and probe enzyme specificity.[7–9] This strategy is outlined in Scheme 1, and entails the introduction of a cysteine residue at a key active site position via site-directed mutagenesis whose side chain is then thioalkylated with an alkyl methanethiosulfonate reagent (**1a–d**) to yield a chemically modified mutant enzyme (CMM) of the serine protease subtilisin *Bacillus lentus* (SBL).[10–14]

Mutagenesis to cysteine and chemical modification of the S_1 pocket Ser166 residue of SBL was chosen for this study. Previously, the S_1 binding pocket properties of the S166C CMMs of SBL were probed using a series of boronic acid transition state analogue competitive inhibitors.[9,15] The inhibitor probing approach revealed substantial changes in the S_1 binding pocket and also yielded insights into the molecular basis for these changes.[15] Since X-ray crystal structures of the boronic acid inhibitors bound to this and other CMMs are not available, we considered the strategy of employing an active site directed photoaffinity label to map SBL's binding site in order to further investigate the altered binding modes of boronic acid inhibitors to the S_1 pocket.

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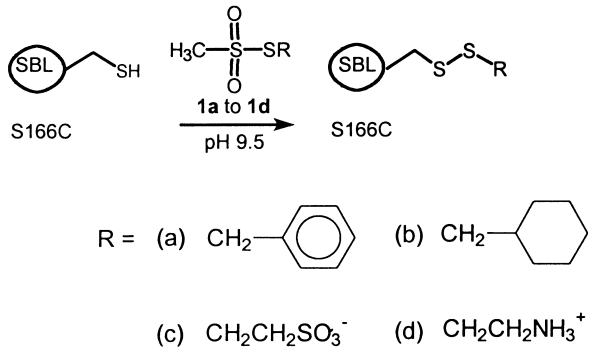
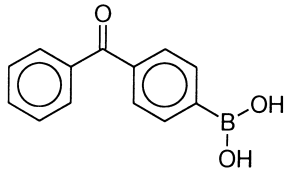


Scheme 1.

Active site directed photoaffinity labels[16–20] have been employed extensively to probe enzyme active sites,[21,22] and to provide structural information complementary to that which may be available from X-ray crystallography[23] or NMR[24,25] approaches. Benzophenone (BP) inhibitor[21,22] and substrate[26] derivatives have been employed extensively in active-site directed photoaffinity labeling studies.[17,18–27] For the current study, the photolabile *p*-boronic acid benzophenone (BBP, **1**) was designed to incorporate the boronic acid transition state analogue inhibitor structure.[28,29–34] The BBP photoprobe has the active site-directing phenyl boronic acid inhibitor incorporated into the structure of BP rather than tethered to the end of the photoprobe, thereby reducing nonspecific labeling complications.[18]



BBP, **1**

The binding pocket topographies of four representative S166C CMMs and of the WT-SBL were investigated. Two of these, S166C-S-$CH_2C_6H_5$ (-**a**) and S166C-S-CH_2-*c*-C_6H_{11} (-**b**) have a sterically constricted S_1 pocket while S166C-S-$CH_2CH_2SO_3^-$ (-**c**) and S166C-S-$CH_2CH_2NH_3^+$ (-**d**) have a negatively and a positively charged S_1 pocket respectively (Scheme 1). In this study we report the synthesis of the BBP reagent, its evaluation as a competitive reversible inhibitor of WT-SBL and of S166C CMMs (Scheme 1), and photolysis of the corresponding BBP–EI complexes.

Results and Discussion

Preparation of CMMs

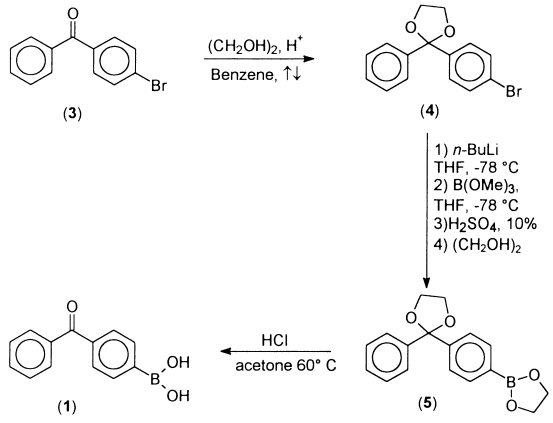
Each of the chemically modified mutant enzymes (CMMs) was prepared[9] and characterized[9,35] by the general method described previously, yielding S166C-S-**a** to -**d** as outlined in Scheme 1.

Preparation of *p*-boronic acid benzophenone

The *p*-boronic acid benzophenone (BBP, **1**) photoprobe designed for the current study was prepared as outlined in Scheme 2. Briefly, 4-bromobenzophenone (**3**) was protected as the glycal in refluxing benzene.[36–37] The glycal (**4**) was then subjected to halogen–lithium exchange by reaction with *n*-butyl lithium, followed by treatment with trimethyl borate to generate the corresponding dimethyl borate.[31,37] To facilitate purification,[31] the dimethyl borate was subsequently deprotected in sulfuric acid and reprotected with ethylene glycol, generating the diglycal (**5**), which was recrystallized from benzene. The purified diglycal (**5**) was then fully deprotected in refluxing 6 M HCl,[37] yielding the *p*-boronic acid benzophenone (BBP, **1**) in 77% overall yield (Scheme 2).

Evaluation of competitive inhibition by BBP

Initially, the efficacy of binding of the designed photolabile competitive inhibitor, BBP, to each of the WT, S166C-S-$CH_2C_6H_5$, S166C-S-CH_2-*c*-C_6H_{11}, S166C-S-$CH_2CH_2NH_3^+$ and S166C-S-$CH_2CH_2SO_3^-$ enzymes was determined by measuring the catalytic activity using suc-AAPF-pNA as substrate.[38] The kinetic data are summarized in Table 1 and, as expected, in all cases BBP binds best to WT. BBP binding to S166C-$SCH_2C_6H_5$ (-**a**) and S166C-CH_2-*c*-C_6H_{11} (-**b**), with their large hydrophobic side chains, is reduced by 86-fold and 9-fold, respectively, compared to WT. Previously,



Scheme 2.

Table 1. Kinetic and inhibition constants for S166C CMMs with BBP

Enzyme	K_M mM	k_{cat} s^{-1}	k_{cat}/K_M mM^{-1} s^{-1}[a]	K_I mM[b]
WT	0.55 ± 0.06	48 ± 2	87 ± 10	0.037 ± 0.004
S166C-S-**a**	0.74 ± 0.09	6.9 ± 0.4	9 ± 1	3.2 ± 0.04
S166C-S-**b**	0.61 ± 0.04	8.7 ± 0.2	14.3 ± 0.9	0.32 ± 0.02
S166C-S-**c**	0.70 ± 0.06	3.8 ± 0.1	5.4 ± 0.5	6.3 ± 0.6
S166C-S-**d**	0.60 ± 0.06	16.3 ± 0.5	27 ± 3	0.15 ± 0.02

[a]Michaelis–Menten constants were measured by the initial-rate method in 0.1 M phosphate buffer (pH 7.5, 0.5 M NaCl) at 25 °C with suc-AAPF-pNA as the substrate.
[b]Inhibition constants were determined under the same conditions by the method of Waley.[39]

all the boronic acids evaluated[15] exhibited poorer binding with the S166C-S-$CH_2C_6H_5$ CMM compared to WT, and therefore the increased K_I of BBP with this CMM is not unexpected. That BBP binding to S166C-S-CH_2-*c*-C_6H_{11} is better than to S166C-S-$CH_2C_6H_5$ may be due to the greater hydrophobicity of its side chain or to differences in side chain orientation. BBP binding to the charged CMMs, S166C-S-$CH_2CH_2SO_3^-$ (**-c**) and S166C-S-CH_2 $CH_2NH_3^+$ (**-d**), is reduced 170-fold and 4-fold respectively compared to WT. This inhibition pattern is comparable to that revealed by the previous boronic acid inhibitor screens, for which all except for the smallest inhibitor, phenyl boronic acid, exhibited poorer binding to S166C-$CH_2CH_2SO_3^-$ compared to WT.[15] In contrast, the apparently more accommodating nature of the S_1 pocket of S166C-$CH_2CH_2NH_3^+$ is in accord with the fact that three of the five boronic acid inhibitors evaluated exhibited improved binding compared to WT.[15] Thus, BBP binding to SBL exhibits the same activity patterns as did the previously evaluated boronic acid transition state analogue inhibitors.

Table 2. ESMS data for BBP treated WT-SBL

Treatment	Average mass calculated	Average mass found	Interpretation
hv	26698	26702	WT enzyme
2× BBP + *hv*	26888	26888	WT + BBP $-2H_2O$
10× BBP + *hv*	26888	26891	WT + BBP $-2H_2O$
2× BBP + dark	26698	26702	WT enzyme
10× BBP + dark	26698	26706	WT enzyme

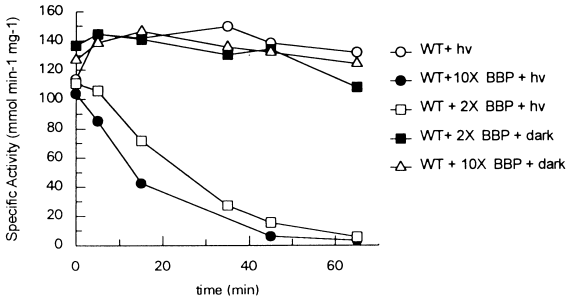


Figure 1. Time course for photolysis (350 nm) of SBL-WT and BBP.

Photolysis of enzyme–BBP complexes

The advantages of the BP photophore have been documented and include its chemical stability, its stability to ambient light and its 330–360 nm photoactivation range, which is not damaging to proteins.[18] In addition, BP is particularly well suited to the current probing study since SBL's active site is quite hydrophobic and BP exhibits preferential labeling of hydrophobic binding regions in proteins.[18] The photolysis of benzophenone derivatives at λ 320–360 nm effects an *n* to π* transition which induces formation of a covalent link to amino acid residues, or other functionalities, by H-abstraction and radical recombination.[17,18–27]

The initial photolysis time-course experiments were done with WT-SBL. The photolysis reactions were conducted at 4 °C in slightly acidic buffer (pH 5.8) in order to reduce autolysis of the enzyme. The BBP photoprobe was used at both a 2-fold and a 10-fold molar excess concentration with respect to the enzyme. Control and blank reactions were conducted under the same conditions. Aliquots were withdrawn and specific activity measurements made.

The photolysis time-course experiment revealed a time dependent decrease in specific activity falling off to zero after 60 min (Fig. 1). Both the 2-fold and 10-fold molar excess of BBP reactions effected the same final activity, with the more concentrated reaction mixture resulting in a slightly faster activity loss. For both the WT photolyzed control (WT + *hv*) and the dark control (WT + BBP) reactions, activity was unaltered. This established that the activity losses observed were due exclusively to the photochemical reaction between the enzyme and BBP. After photolysis, each sample was purified and analyzed by ESMS. Both the WT photolyzed (WT + *hv*) control and the dark (WT + BBP + dark) control retained the original WT mass, demonstrating that no chemical changes were induced by photolysis of the enzyme in the absence of BBP nor by treatment of the enzyme with BBP in the dark. However, for the WT enzyme photolyzed in the presence of either a 2-fold or a 10-fold molar excess of BBP a mass increase consistent with covalent linkage of BBP followed by the loss of two moles of water, was observed, as summarized in Table 2.

To identify the location of the crosslink of BBP to the enzyme, peptide mapping analysis was employed.[40] Specifically, each of the enzyme samples was denatured first by acid then by urea and then degraded by trypsin into fragments designated 1T to 13T, which correspond to the tryptic fragments numbered sequentially from the amino terminus of the enzyme. These fragments were then separated by reverse-phase HPLC which, coupled to mass spectrophotometric analysis, permitted the identification of each of the tryptic fragments on a peptide map.[40] Comparison of the mass and retention time of the BBP-treated and photolyzed enzyme to that of the control was used to identify the BBP modified peptide fragment. This revealed a modification on tryptic fragment 6T, which was comprised of amino acids 93–143 in the linear sequence. Modification resulted in the disappearance of fragment 6T and yielded two new peaks, a minor peak designated as 6T′ and a major peak designated as 6T″. Both of these were more hydrophobic than 6T itself, as evidenced by their longer retention times on reverse-phase HPLC, and showed mass increases over 6T of 197 and 189 Da for 6T′ and 6T″, respectively (Fig. 2).

The peptides 6T′ and 6T″ corresponded to the BBP modified tryptic fragment 6T and were isolated by HPLC. The specific location of the covalent bond between BBP and the 6T′ and 6T″ tryptic fragments were identified by automated amino acid sequencing of the isolated peptides. For the minor peptide 6T′ the Edman sequencing signal became weak after cycle 33

revealing the sequence VLGASGSGSVSSIAQGLE-WAGNNGMHVANLS^{126}L^{127}**G**^{128}xxx (BPN′ numbering). However, it was not clear if the cycle stopped at this point, or just weakened. In contrast, for the major peptide 6T″ an abrupt termination after cycle 32 was noted, revealing the sequence …VANLS^{126}**L**^{127}xxx (BPN′ numbering). The sequence information obtained for the major peptide 6T″ identified a modification at residue Gly127 (BPN′ numbering). This is highly significant since Gly127 is located on the back wall of the S_1 pocket. A proposed crosslinking mechanism which accounts for this modification, and for the loss of two water molecules, and which is consistent with the observed mass change, is presented in Figure 3. The modification site of the minor peptide fragment 6T′ is not conclusive but may be at residue Ser128.

The above results demonstrate the validity of the approach for probing the active site of WT-SBL. Accordingly, the same protocol was applied to the CMMs S166C-S-**a** to -**d**, using a 10-fold molar excess of BBP, in order to probe BBP binding changes. However, despite a 12 h photolysis time, specific activity was not decreased to zero for any of the CMM samples. Nonetheless, each of the photolyzed CMMs and the dark controls, were purified and analyzed by ESMS. For each of S166C-S-**a**, -**c**, -**d**, no modification was indicated by ESMS (Table 3). However, a mass increase of 220 Da was observed for S166C-S-CH_2-*c*-C_6H_{11} (-**b**) 10×BBP + *hv*, which is consistent (±6 Da) with the formation of a covalent bond between S166C-S-**b** and BBP (calculated mass of BBP 226 Da).

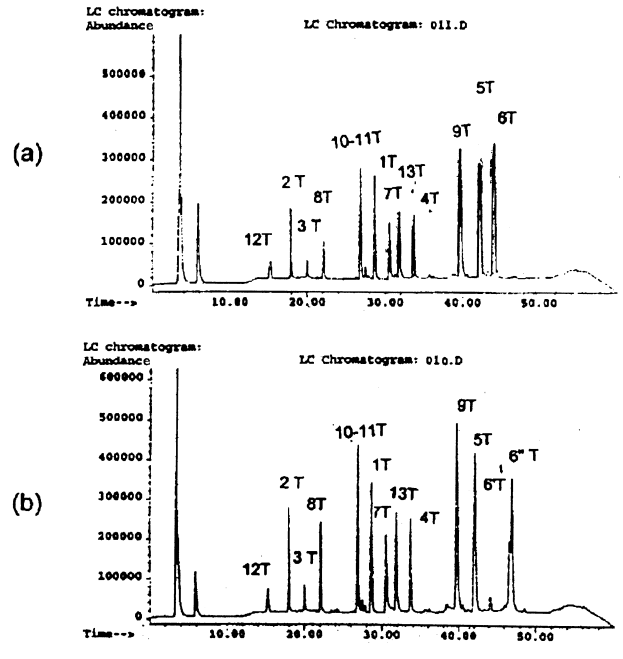


Figure 2. HPLC-trace of (a) native and (b) BBP crosslinked WT-SBL: tryptic fragments 1T—13T are identified.

Table 3. ESMS data for 10×BBP treated S166C-S-**x**

S166C-S-x/ treatment	Average mass calculated	Average mass found	Interpretation
-**a** BBP + dark	26836	26847	Unchanged CMM
-**a** BBP + *hv*	27062	26839	Unchanged CMM
-**b** BBP + dark	26842	26844	Unchanged CMM
-**b** BBP + *hv*	27068	27062[a]	CMM + BBP[a]
-**c** BBP + dark	26853	26868	Unchanged CMM
-**c** BBP + *hv*	27079	26867	Unchanged CMM
-**d** BBP + dark	26790	26818	Unchanged CMM
-**d** BBP + *hv*	27016	26818	Unchanged CMM

[a]Poorly resolved spectrum.

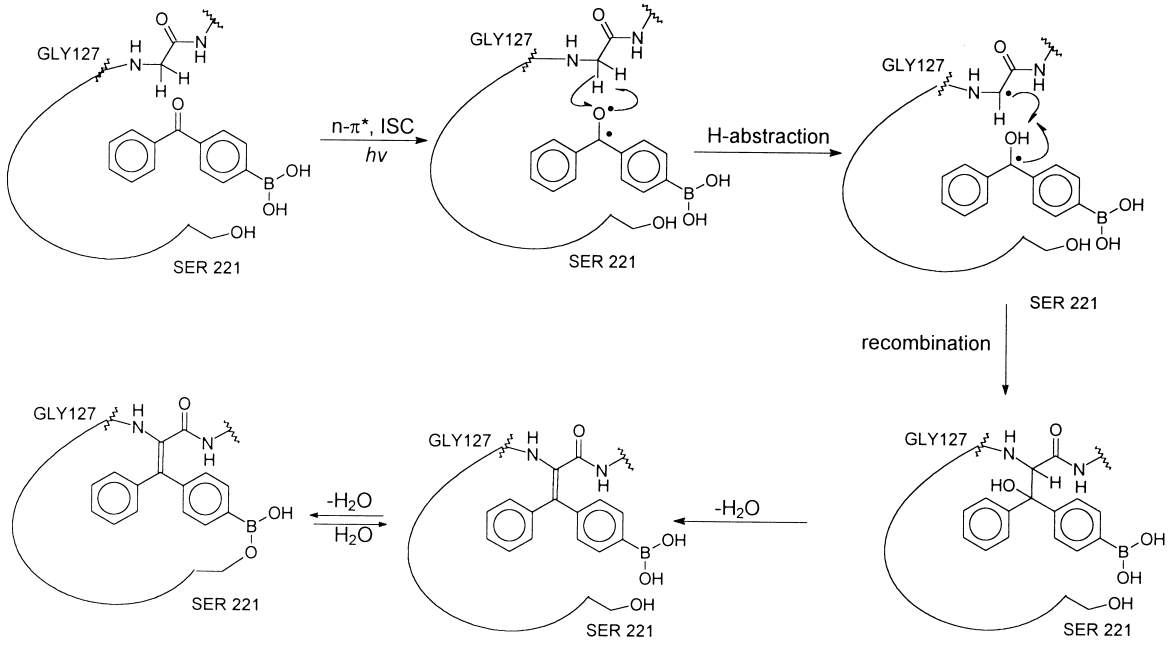


Figure 3. Proposed mechanism of BBP crosslinking: the photoactivated crosslinking of BBP to Gly127 and dehydration. The last dehydration step forming a boronate ester between BBP and the enzyme may precede crosslinking.

For the S166C-S-*c*-C_6H_{11} CMM, both the dark control (S166C-S-**b** 10×BBP + dark) and photolyzed sample (S166C-S-**b** 10×BBP + *hv*) were subjected to tryptic digestion and peptide mapping, as described above for the WT enzyme. For the photolyzed S166C-S-**b** (10×BBP + *hv*) sample a reduction in intensity of the tryptic fragment 6T (50%) was observed which was accompanied by two new peaks, a minor peak designated as 6T′ and a major peak designated as 6T″. Both 6T′ and 6T″ were more hydrophobic than 6T itself and showed mass increases over 6T of 197 and 188 Da respectively. The tryptic fragments 6T′ and 6T″ were isolated and subjected to amino acid sequencing. The sequences for 6T′ and 6T″ were identical. The observed …VANLS^{126}**L**^{127}xxx (BPN′ numbering) sequence was identical to that derived from the 6T″ tryptic fragment of the WT-SBL + BBP photolyzed sample. The observed mass increase of 188 Da (BBP-2H_2O:190 Da calcd) for the tryptic fragment, is consistent with the formation of a crosslink between the S166C-S-**b** and BBP with concomitant loss of two water molecules. However, it must be noted that the mass of the intact S166C-S-**b**–BBP complex does not demonstrate the concomitant loss of two molecules of water. This is attributable to the poorly resolved mass spectrum in this case (Table 3). The identity of the minor tryptic fragment 6T′ is not apparent. Both the WT and S166C-S-CH_2-*c*-C_6H_{11} enzymes show a crosslink to BBP at residue Gly127 despite having very different active site environments. This suggests that BBP forms a boronate ester adduct with the enzyme, which although reversible is sufficiently long-lived that it acts as a tether restricting the spatial span of the BBP reagent in the enzyme active site. Such adducts between boronic acid inhibitors and the Ser221 Oγ hydroxyl of serine proteases have been observed previously by X-ray crystallography[33] and by NMR.[41]

Since no crosslinks were observed for S166C-S-**a**, **-c**, **-d** after photolysis for 12 h in the presence of 10-fold molar excess of BBP, each of these CMMs were photolyzed for up to 72 h in the presence of a 80-fold molar excess of BBP (the solubility limit). However, although activity decreases were effected under these conditions, purification of the enzymes followed by their ESMS analysis did not provide evidence of crosslinking. Thus it was concluded that the introduced side chains of S166C-S-**a**, **-c** and **-d** CMMs preclude proper orientation or alignment of BBP in the S_1 pocket for crosslinking with the enzyme. The significantly reduced binding of BBP to S166C-S-**a** and **-c,** and the slightly reduced binding of BBP to S166C-S-**b** and **-d**, as manifested by higher K_I values, and the lack of an observed crosslink between BBP and S166C-S-**a**, **-c** and **-d** and the longer reaction time, compared to WT, required to effect a crosslink between S166C-S-**b** and BBP, indicate that modification of residue C166 makes the S_1 pocket less conducive to binding large P_1 groups. It is interesting that despite the fact that BBP binding to S166C-S-**b** is 2-fold better than to S166C-S-**d**, the former does not form a BBP crosslink while the latter does. The reasons for this effect are unknown and intriguing.

Experimental

Preparation of *p*-boronic acid benzophenone (1). A stirred solution of 4-bromobenzophenone (3.12 g, 12.0 mmol), ethylene glycol (4.7 g, 75 mmol), and *p*-toluene sulfonic acid (0.2 g, 1.2 mmol) in benzene (30 mL) was heated under reflux for 48 h and the resulting water was removed by azeotropic distillation in a Dean–Stark trap.[36,37] The reaction mixture was allowed to cool to 22 °C and then aqueous 1 M NaOH (40 mL) was added and the mixture was extracted with Et_2O (3×30 mL). The organic phases were combined, dried ($MgSO_4$), filtered, and then evaporated *in vacuo* yielding 4-bromobenzophenone ethylene glycol (**2**) as a white solid which was recrystallized from EtOH (3.7 g, 12 mmol, quantitative yield).[36] ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.03 (4H, s, CH_2), δ 7.27–7.50 (9H, m, Ar-H) ppm.

To a stirred solution of 4-bromobenzophenone ethylene glycol (**3**), (1.0 g, 3.28 mmol) dissolved in dry THF (10 mL) under N_2 at −78 °C was added drop wise via syringe *n*-butyl lithium (1.7 mL of a 2.5 M solution in hexane, 4.3 mmol).[37] This mixture was stirred for 1 h then transferred by cannula to a solution of trimethylborate (0.51 g, 4.9 mmol) in dry THF (10 mL) at −78 °C.[31,37] This mixture was stirred at −78 °C for 1 h and then allowed to warm to room temperature overnight. The reaction mixture was slowly added to aqueous 10% H_2SO_4 (20 mL) at 0 °C and stirred for 30 min.[31] The reaction mixture was then extracted with Et_2O (3×30 mL). The combined organic phases were dried ($MgSO_4$), filtered and then evaporated in vacuo to yield the boronic acid as a yellow oil which was added directly to a flask containing a solution of ethylene glycol (0.2 g, 3.3 mmol) in Et_2O (25 mL) and stirred vigorously for 1 h at 20 °C.[31] To this reaction mixture were added hexanes (50 mL) and water (1 mL) and the aqueous layer was separated. The organic layer was dried ($MgSO_4$), filtered, and then evaporated in vacuo yielding the diglycal (**4**) as a white solid which was recrystallized from benzene (0.87 g, 2.9 mmol, 88% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.06 (4H, s, CH_2), 4.36 (4H, s, CH_2), 7.27–7.50 (9H, m Ar-H) ppm.

The diglycal **4**, (270 mg, 0.91 mmol) was dissolved in a stirred solution of 6 M HCl (15 mL) and acetone (5 mL) and heated to 60 °C for 2 h with stirring and then stirred for a further 48 h at room temperature.[37] The reaction mixture was extracted with Et_2O (2×20 mL). The combined organic phases were then washed with brine (20 mL), poured into H_2O (5 mL) and concentrated in vacuo, with the benzophenone boronic acid precipitating as white crystals (180 mg, 0.8 mmol, 87% yield). ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.35 (2H, s, OH), δ 7.58–7.98 (9H, m, Ar-H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6) δ 196.2, 138.2, 137.1, 134.1, 132.8, 129.7, 128.6, 128.5 ppm. IR (KBr) 3500–3200 ($B(OH)_2$), 2924 (aromatic CH) 1654 (carbonyl), 1318, 1284, 1113, 875, 704 cm^{-1}, mp 103 °C. HRMS for trimeric anhydride $C_{39}H_{27}BO_3$ calcd: 624.2086. Found: 624.2095.

Site-specific chemical modification. To 25 mg of the S166C mutant, purified as previously described[9,28] and

stored flash frozen in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5), at 20 °C was added in turn one of the methanethiosulfonate reagents (**2a**–**d**) (100 μL of a 0.2 M solution) in a PEG (10,000) coated polypropylene test tube, and the mixture agitated in an end-over-end rotator. Blank reactions containing 100 μL of solvent instead of the reagent solution were run in parallel. Each of the modification reactions was monitored spectrophotometrically ($\epsilon_{410} = 8800\ M^{-1}\,cm^{-1}$)[42] on a Perkin–Elmer Lambda 2 spectrophotometer, by specific activity measurements. After the reaction was quenched by dilution with MES buffer (5 mM MES, 2 mM $CaCl_2$, pH 6.5) at 0 °C, the specific activity of the CMM (10 μL) was determined in buffer (0.1 M Tris pH 8.6, 0.005% Tween 80 and 1% DMSO, with the suc-AAPF-pNA as substrate (1 mg/mL, purchased from Bachem Bioscience Inc.) at 25 °C. The reaction was terminated when the addition of a further 100 μL of methanethiosulfonate solution effected no further change in specific activity, generally within 30 min. The reaction solution was purified on a disposable desalting column (Pharmacia Biotech PD-10, Sephadex G-25 M) pre-equilibrated with MES buffer (5 mM MES, 2 mM $CaCl_2$, pH 6.5) then dialyzed against 20 mM MES, 1 mM $CaCl_2$, pH 5.8 (3×1 L) at 4 °C and aliquoted into 0.5–1.5 mL volumes, flash frozen in liquid nitrogen and then stored at −20 °C. Modified enzymes were analyzed by nondenaturing gradient (8–25%) gels at pH 4.2, run towards the cathode on the Pharmacia Phast-System according to Pharmacia Method 300, and appeared as one single band.

Electrospray mass spectrometry. The CMMs were purified for ESMS analysis, by FPLC (BioRad, Biologic System) on a Source 15 RPC matrix (17-0727-20 from Pharmacia) with 5% acetonitrile, 0.01% TFA as the running buffer and eluted with 80% acetonitrile, 0.01% TFA in a one step gradient. Electrospray mass spectra were recorded on a PE SCIEX API III Biomolecular Mass Analyzer. Mass: WT. Calcd: 26698. Found: 26694. S166C-S-**a**: calcd: 26836. Found: 26832. S166C-S-**b**: calcd: 26842. Found: 26844. S166C-S-**c**: calcd: 26853. Found: 26851. S166C-S-**d**: calcd: 26714. Found: 26708

Regeneration of unmodified enzyme by treatment with β-mercaptoethanol. To a solution of CMM (2.0 mg) in 250 μL of CHES-buffer (70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) was added 10 μL of a solution of β-mercaptoethanol (1 M in 95% EtOH). The reaction was monitored by specific activity measurements and in all cases the activity of the S166C cysteine parent was restored.

Free thiol titration. The residual free thiol content of the S166C CMMs, was determined spectrophotometrically by titration with Ellman's reagent ($\epsilon_{412} = 13{,}600\ M^{-1}\,cm^{-1}$)[35] in sodium phosphate buffer (0.25 M, pH 8.0).

Active site titration. The active enzyme concentration was determined as previously described[43] by monitoring fluoride release upon enzyme reaction with α-toluenesulfonyl fluoride (Aldrich Chemical Co. Inc.) as measured by a fluoride ion sensitive electrode (Orion Research 96-09). The active enzyme concentration determined in this way was used to calculate kinetic parameters for each CMM.

Photolysis. Enzyme solutions for photolysis were prepared in 3.5 mL quartz cuvettes and contained 3 mL of enzyme dissolved (2 mg/mL) in storage buffer (20 mM MES, 1 mM $CaCl_2$, pH 5.8). To each cuvette was added 10 μL of *p*-boronic acid benzophenone (for 10×samples, 0.02 M BBP in DMSO). For the blank reactions 10 μL of DMSO was added instead of BBP. The solutions were photolyzed at 4 °C in a Rayonet photoreactor equipped with 350 nm lamps. The dark controls were treated in the same way except the cuvettes were wrapped in aluminum foil. At the time intervals indicated, 10 μL aliquots were withdrawn, diluted into 190 μL of storage buffer and specific activity measurements made. After photolysis, the enzymes were purified on a desalting column (Pharmacia Biotech PD-10, Sephadex G-25 M) pre-equilibrated with MES buffer (3.5 mL, 5 mM MES, 2 mM $CaCl_2$, pH 6.5). The enzyme was eluted with MES buffer, dialyzed against storage buffer (20 mM MES, 1 mM $CaCl_2$, pH 5.8, (3×1 L)) at 4 °C aliquoted, flash frozen and stored at −20 °C.

Specific activity measurements for time course monitoring. For specific activity determinations, absorbance versus time measurements were made on a Perkin–Elmer Lambda 2 spectrophotometer. The spectrophotometer was zero'd against a 1 mL disposable cuvette containing 980 μL of pH 8.6 0.1 M Tris buffer containing 0.005% Tween 80 and 10 μL of the suc-AAPF-pNA substrate (100.0 mg/mL in DMSO, 160 mM). The enzyme aliquot (10 μL) was added and, after a 10 s delay, the absorbance reading was started and sampled with an interval of 0.2 s during 60 s at λ = 410 nM. The slope (in A s^{-1}) was determined by linear curve fit of the data below 5% of substrate conversion. The rate of product formation, dP/dt was determined using Beer's Law ($A = \epsilon c \lambda$, where $\epsilon_{410} = 8800\ M^{-1}\,cm^{-1}$, $\lambda = 1$ cm). The enzyme activity was then calculated: specific activity (μmol min^{-1} mg^{-1}) = {(dP/dt M s^{-1})(60 s min^{-1})(10^6 μmol mol^{-1}) (volume in cuvette L)}/{[E] mg mL^{-1}}.

Tryptic digests and HPLC-MS analysis.[40] From a stock solution of the SBL samples in storage buffer (20 mM MES, 1 mM $CaCl_2$, pH 5.8) was withdrawn 0.1 mg (≤100 μL) of enzyme which was mixed with water (≥200 μL) and 1 N HCl (30 μL) to give a final concentration of 0.03 mg/mL protein in 0.09 M HCl. This mixture was chilled for 10 min on ice and any debris was removed by centrifugation for 2 min at 14,000 rpm. The supernatant was transferred to a clean Eppendorf tube to which was added 35 μL TCA (50% w/v) to give a final concentration of 5% (w/v) TCA. This mixture was chilled for 15 min on ice and then the protein was collected by centrifugation (2 min at 14,000 rpm). The pellet was washed once with 1 mL of 90% (v/v) acetone (−20 °C) and briefly dried in vacuo. The pellet was resuspended in 12 μL of 0.5 M ammonium bicarbonate, containing 8 M urea and then incubated at 37 °C for 4 min. To this mixture was added 48 μL of 1% (w/v

in H_2O) 1-*O*-octyl-β-D-glucopyranoside and 1 μL of trypsin (Sigma Chemical Co. TPCK-treated, 2.5 mg/mL in 1 mM HCl) and then the mixture was at 37 °C for 15 min after which time the reaction was terminated by adding 5 μL of TFA (10% v/v).

An aliquot of the tryptic digest was then analyzed on an Hewlett–Packard HPLC Model 1090, which is equipped with a Vydac C-18 reverse phase column Model 218T952 (2.1×250 mM), with a gradient of water +0.1% TFA and CH_3CN + 0.1% TFA as the mobile phase, coupled to a Hewlett–Packard electrospray ionization mass spectrophometer Model 59987B.

Peptide sequencing. The isolated tryptic fragments were sequenced on the applied biosystems Model 473A protein sequencer.

Kinetic measurements. Michaelis–Menten constants were determined at 25 °C by curve fitting (GraFit® 3.03) of the initial rate data determined at eight concentrations (0.125–4.0 mM) of the suc-AAPF-pNA substrate ($\epsilon_{410} = 8800\ M^{-1}\,cm^{-1}$)[42] in 0.1 M phosphate buffer containing 0.5 M NaCl, 0.005% Tween 80, 1% DMSO, pH 7.5.

Determination of *p*-boronic acid benzophenone inhibition constants. K_I values for BBP were determined in duplicate by the method of Waley.[39] The progress curve without inhibitor was determined from an assay mixture containing 980 μL of buffer (0.1 M phosphate buffer containing 0.5 M NaCl, 0.005% Tween 80, pH 7.5), 5 μL DMSO and 5 μL of suc-AAPF-pNA substrate (50 mM in DMSO). This mixture was incubated in a water jacketed cell for 5 min at 25 °C and the absorbance reading was set to zero prior to initiating the reaction by addition of 10 mL of enzyme solution (3.2×10^{-6} to 1.1×10^{-4} M in pH 5.8, 20 mM MES, 1 mM $CaCl_2$). The final volume of the assay mixture was 1 mL. The progress curve with inhibitor was determined similarly but with 5 μL of the BBP inhibitor solution (7.24×10^{-3} to 1.4×10^{-1} M, in DMSO) being added instead of 5 mL DMSO, to a final volume of 1 mL. Absorbance versus time measurements were recorded on a Perkin–Elmer Lambda 2 spectrophotometer and points for calculation were taken at 15, 18, 21, 24, 27, 30, 33 and 36% substrate conversion.[44]

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