Formation of a Trypsin−Borate−4-Aminobutanol Ternary Complex

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ABSTRACT: The formation of ternary complexes involving serine proteases, borate, and an alcohol has important implications for understanding the physiological actions of borate and for the development of tight binding inhibitors for this class of enzymes. Recent studies of a related enzyme, γ-glutamyl transpeptidase, which is subject to inhibition by a labile serine/borate mixture, have demonstrated that construction of a nonlabile boronate analogue results in an inhibitor with nearly 10^5-fold greater potency. To evaluate the generalization of this biochemistry to serine proteases, we have observed the ternary conplex formed from 4-aminobutanol, borate, and trypsin. A combination of ^{11}B and ^{1}H NMR and spectrophotometric assays using acetylarginine *p*-nitroanilide (Ac-Arg-pNA) as the chromogenic substrate all indicate a cooperative binding interaction in which the borate is esterified by the oxygen atoms of the 4-aminobutanol and trypsin residue Ser^{195}. Two downfield-shifted proton resonances at 15.5 and 16.6 ppm are proposed to arise from the labile imidazolium protons on His^{57}, indicating a salt bridge interaction with the negatively charged borate. A cooperativity parameter α of 0.2 is derived from the assays. These results provide the first direct evidence for formation of a ternary complex involving a serine protease, borate, and an alcohol, and suggest that this represents a general approach for the development of tight binding ligands.

Boric acid has been implicated in a range of biochemical and physiological phenomena (*1*, *2*), and exhibits selective toxicity to male reproductive function (*3*). One of the most interesting aspects of borate biochemistry is the dramatic, cooperative inhibition of the enzyme γ-glutamyl transpeptidase (γ-GT)[1] by a serine/borate mixture, discovered initially due to the use of borate as a high-pH buffer (*4*). This effect was subsequently proposed to result from the ability of this enzyme to synthesize or select its own inhibitor by stabilizing a labile borate−serine ester in the active site (*5*). This inhibitor, a structural transition state analogue, involves a tetrahedral borate esterified both to an active site hydroxyl residue (presumably Thr^{391}; *6*) and to the hydroxyl group of the serine, which also binds to the active site. This proposal leads to several interesting predictions. First, as we have recently demonstrated, L-2-amino-4-boronobutanoate, a structural analogue of the labile serine−borate complex, is found to be a strong inhibitor of γ-GT, with a K_i of 17 nM (*7*).[2] This value is more than 4 orders of magnitude lower than the K_i for a serine/borate mixture, reported to be 1.45 mM (*8*). Although γ-GT is a rather unusual enzyme, its biochemical mechanism appears to be similar to that of the serine proteases. Thus, it would seem possible that in general, analogous ternary complexes involving serine proteases, borate, and alcohols can form as well. The existence of such complexes has significant implications for understanding the biochemical and physiological effects of borate, as well as for the design of strong inhibitors for this class of enzymes, as previously demonstrated for the case of γ-GT.

To test this more general hypothesis, we have investigated whether evidence for a ternary alcohol−borate−enzyme complex can be demonstrated for the serine protease trypsin. For this class of enzymes, it has been known for some time that potent inhibitors can be developed by replacing the α-carboxyl group of a C-terminal product peptide derived from the hydrolytic reaction with a structurally analogous boronic acid function (*9*−*11*). Upon complexation with trypsin, the boronic acid forms a tetrahedral structure resulting from a nucleophilic addition by Ser^{195} in the active site. As a result of the P1 selectivity of trypsin, these boronates most typically utilize boronic acid analogues of lysine or arginine as the C-terminal residue. On the basis of the reasoning outlined above, these results imply that structurally related ternary complexes composed of borate and 4-aminobutanol in the first case, or guanidino-3-propanol in the second, should be stabilized or synthesized in the active site of trypsin.

MATERIALS AND METHODS

Type IX porcine pancreatic trypsin was obtained from Sigma (St. Louis, MO), and other chemicals were from Aldrich and used without further purification. ^{1}H and ^{11}B NMR spectra were obtained on a Varian Unity 500 NMR spectrometer operating at 160.6 MHz for boron. The ^{1}H spectra were obtained at 5 °C and utilized a jump and return sequence to suppress the H_2O resonance (*12*). The boron spectra were obtained at 25 °C using 5 mm quartz NMR

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[1] Abbreviations: Ac-Arg-pNA, acetylarginine *p*-nitroanilide; γ-GT, γ-glutamyl transpeptidase; NMR, nuclear magnetic resonance.

[2] This inhibitor also has been subsequently identified by Stein and co-workers (*43*).

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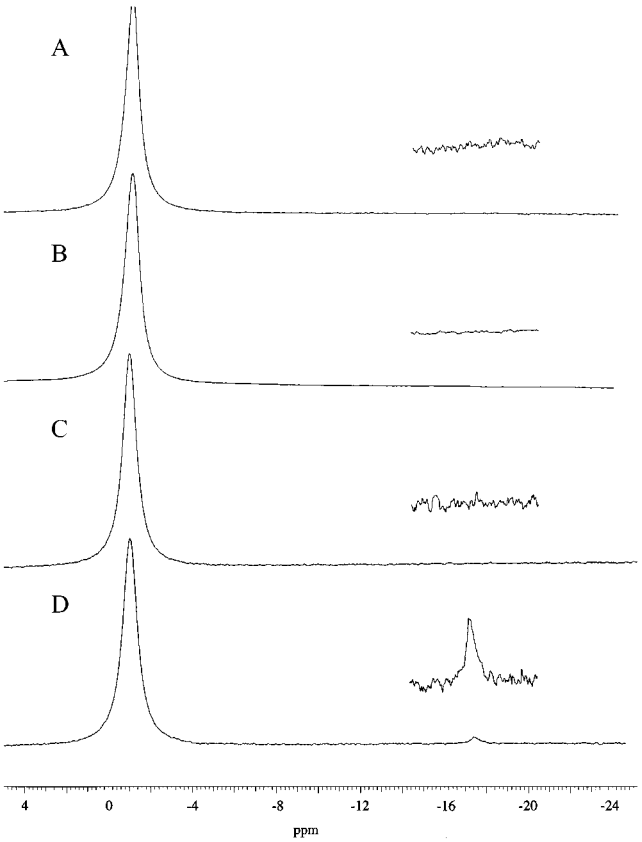


FIGURE 1: ^{11}B NMR spectra obtained at 160.6 MHz and 25 °C on samples containing (A) 5 mM borate and 2.0 mM porcine pancreatic trypsin (type IX, from Sigma), (B) 25 mM borate and 250 mM 4-aminobutanol, (C) 5.0 mM borate, 50 mM 3-aminopropanol, and 2.0 mM trypsin, and (D) 5.0 mM borate, 50 mM 4-aminobutanol, and 2.0 mM trypsin. Insets show a 10-fold vertical expansion. All samples were made up in 20 mM HEPES buffer (pH 8.0). Samples were run on a Varian Unity 500 NMR spectrometer using a 5 mm Nalorac NMR probe modified to remove borate background signals, and using quartz NMR tubes (Wilmad). Other spectral parameters were as follows: sweep width, 10 kHz; acquisition time, 0.65 s; 100 000 transients. ^{11}B shifts are referenced to external boric acid at pH 4.0.

tubes (Wilmad, Buena, NJ), and using a 5 mm Nalorac probe (Martinez, CA), modified to reduce the boron background. Spectrophotometric assays were performed on a Beckman DU 640 spectrophotometer using 0.3 mM acetylarginine *p*-nitroanilide (Ac-Arg-pNA) as a chromogenic substrate and 200 nM porcine pancreatic trypsin and measuring the absorbance at 410 nm (*13*, *14*). Kinetic data were analyzed using Mathematica (Wolfram Research, Champaign, IL). The program was based on the equation given in the text.

RESULTS

Trypsin assays and ^{11}B NMR (*15*−*18*) data provide the most direct way of assessing the possible formation of a ternary complex. A series of ^{11}B NMR spectra obtained for the proposed ternary complex and related control samples is shown in Figure 1. Binary samples containing a combination of 2.0 mM trypsin and 5 mM borate (Figure 1A) or 250 mM 4-aminobutanol and 25 mM borate (Figure 1B) exhibit an intense boric acid/borate resonance at −1.0 ppm, referenced to external boric acid at pH 4.0, but no resolved upfield resonance which would be expected for a slowly exchanging tetrahedral borate complex (*19*, *20*). In contrast, an ^{11}B resonance at −17.3 ppm is readily observed in a sample containing 2.0 mM trypsin, 5 mM borate, and 50 mM 4-aminobutanol (Figure 1D). As another control, we obtained an ^{11}B spectrum for a sample prepared as described for Figure 1D, but containing 3-aminopropanol instead of 4-aminobutanol (Figure 1C). Again, no separate resonance that can be attributed to a tetrahedral borate complex is observed. These data provide strong support for the conclusion that a ternary trypsin−borate−4-aminobutanol complex forms, characterized by an upfield-shifted ^{11}B resonance. The tetrahedral sp^3 geometry of the borate is indicated by the upfield shift, which is similar to that of $B(OH)_4^-$. Thus, the ternary complex is a transition state mimic, analogous to the structures formed by boronate inhibitors. The enhanced lifetime of borate in the complex, leading to slow NMR exchange behavior, results from formation of a multidentate interaction involving the hydroxyl function of the 4-aminobutanol as well as the active site serine and/or histidine of the trypsin (Scheme 1). There is an extensive literature on the stabilization and resulting slow exchange behavior of sp^3 borate complexed with multidentate ligands (*19*, *20*). We note here that borate by itself is known to inhibit trypsin ($K_i \sim 80$ mM as determined here). However, as with the hydroxyl ion equilibrium, the exchange with the enzyme, presumably involving a complex with His^{57} (*21*), is fast under the conditions studied, and does not produce a separate resonance. At substantially higher borate concentrations, an additional, narrower high-field resonance near −17.5 ppm is observed in the presence of trypsin or trypsin with 4-aminobutanol. This resonance is not affected by the addition of leupeptin and, therefore, appears to arise from complexation with an impurity or with residues not at the active site. However, as is apparent from Figure 1A, the intensity of this adduct is below the detection threshold at 5 mM borate and 2 mM trypsin.

The downfield-shifted imidazole protons of the active site histidine residue in serine proteases can provide useful insight into the nature of the coordination of boronic acid inhibitors (*21*−*25*). ^{1}H NMR spectra of the downfield region of samples containing trypsin and borate or trypsin, borate, and 4-aminobutanol are shown in Figure 2. Saturation of the enzyme with 500 mM borate results in a proton resonance at 15.7 ppm, in good agreement with the value reported by Tsilikounas et al. (*21*). As discussed in that study, this resonance arises from the $N_{\delta 1}$ proton of His^{57} in a complex in which the boron is directly coordinated by $N_{\epsilon 2}$ of the histidine. In the study presented here, two resonances of similar intensity at 15.5 and 16.6 ppm, as well as a very weak shoulder near 16.8 ppm, are observed for a sample containing trypsin, borate, and 4-aminobutanol. The observation of two proton resonances of approximately equal intensity is most directly interpreted as arising from coordination to active site Ser^{195} (Scheme 1A). The resulting complex stabilizes the imidazolium form of His^{57} so that both the $N_{\delta 1}$ and $N_{\epsilon 2}$ protons can be observed. A salt bridge between the negatively charged borate ester and the positively charged imidazolium increases the p*K* of the latter so that the ^{1}H spectrum remains nearly unchanged in the pH range of 6.0−8.0 (Figure 2). Interestingly, the shifts of the proton resonances differ significantly from those reported for complexes formed between trypsin and boropeptide inhibitors

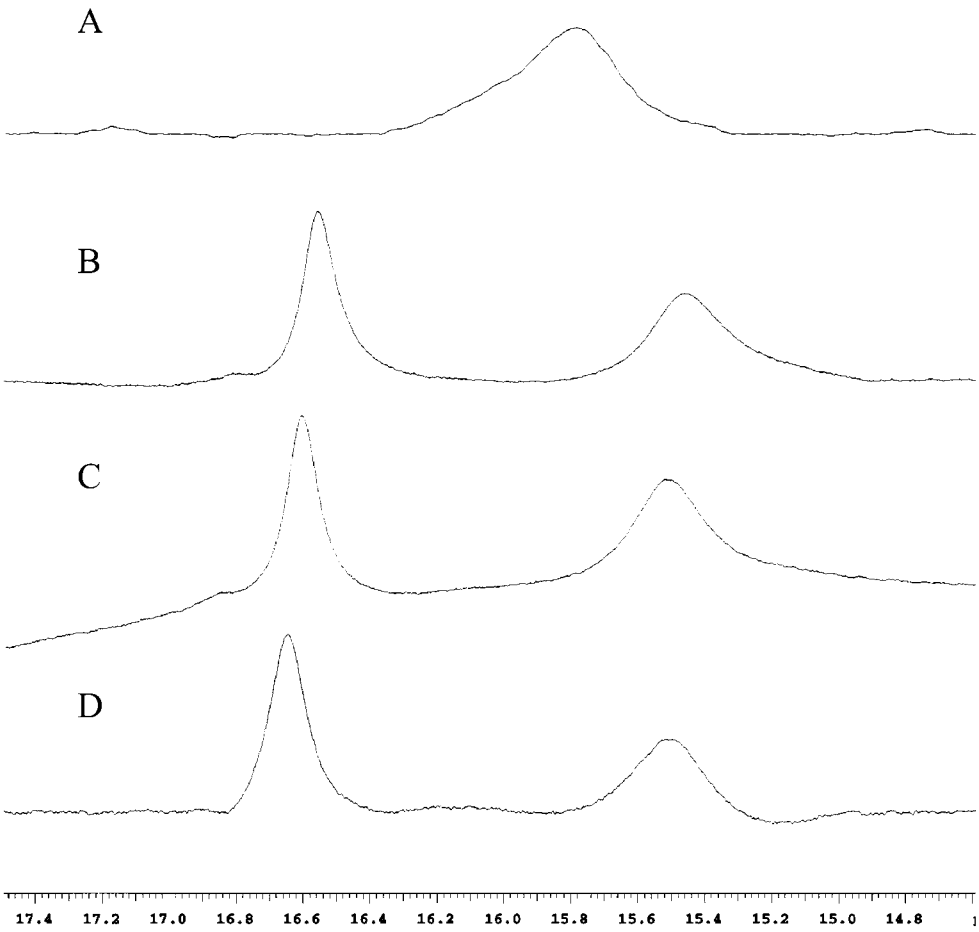
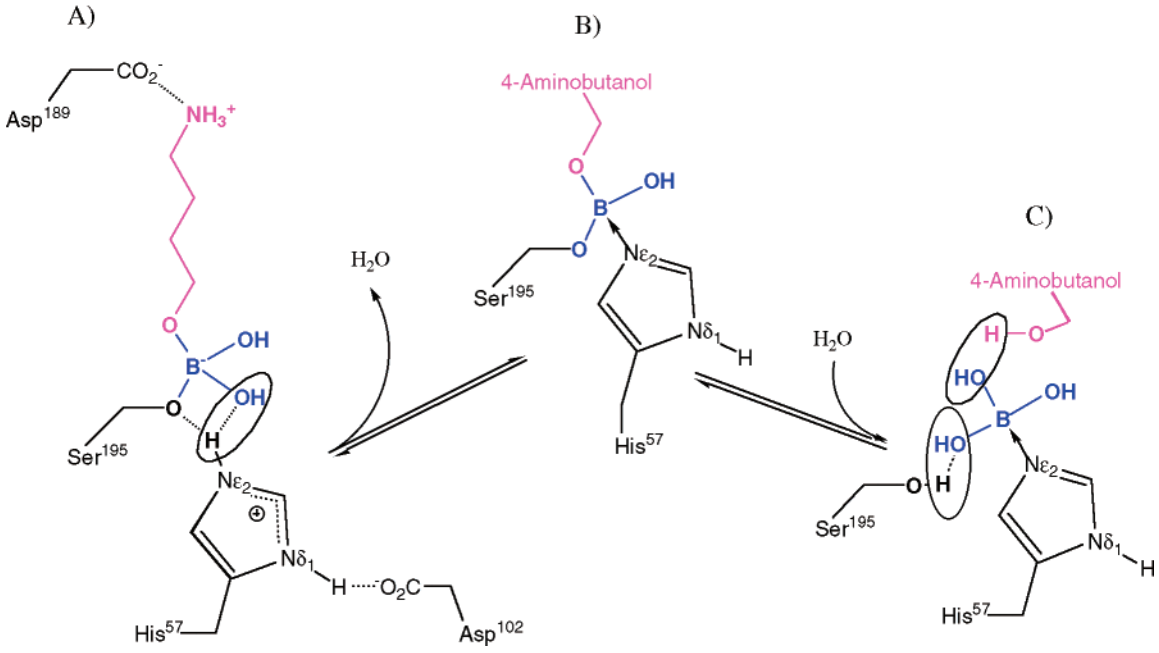


FIGURE 2: Downfield region of the proton NMR spectra of samples containing 2.0 mM trypsin and (A) 500 mM borate, (B) 100 mM borate and 100 mM 4-aminobutanol (pH 8.0), (C) 100 mM borate and 100 mM 4-aminobutanol (pH 7.0), and (D) 100 mM borate and 100 mM 4-aminobutanol (pH 6.0). All samples were run on a Varian Unity 500 NMR spectrometer using a 5 mm proton NMR probe. Proton spectra were obtained at 5 °C using a jump and return water suppression sequence (*12*). Other spectral parameters were as follows: sweep width, 13 kHz; acquisition time, 1.0 s; pulse recycle time, 2.0 s.

Scheme 1



(*21*). In general, the involvement of the histidine $N_{\delta 1}$ and $N_{\epsilon 2}$ protons in hydrogen bonding interactions is expected to significantly affect the observed shift values. Thus, the large shift differences may be indicating a variation in the hydrogen bonding interactions of the $N_{\delta 1}$ and $N_{\epsilon 2}$ protons between complexes of trypsin with the boroArg peptides

studied by Tsilikounas et al. (*21*) and with the 4-aminobutanol borate. The strong sensitivity of these shifts to subtle structural variations is also illustrated by the fact that the assignments of the $N_{\delta 1}$ and $N_{\epsilon 2}$ proton resonances in boropeptide complexes with chymotrypsin and subtilisin are reversed relative to those in α-lytic protease (*24*, *25*). Preliminary analysis of the ^{1}H shifts observed for the ternary complex formed from borate and guanidino-3-propanol indicates values somewhat closer to those of the boropeptides (unpublished results). At this point, we cannot completely rule out the possibility that the observed spectrum corresponds to a mixture of species in which the borate is complexed by the serine and/or histidine residues (Scheme 1). However, in this case, the shifts are equally difficult to rationalize, as is the equal intensity of the two observed peaks. Although a conclusive interpretation of the ^{1}H shits is not possible at this point, the above analysis indicates that the ternary complex involves a borate−Ser^{195} bond, either exclusively (Scheme 1A) or, less probably, as a component of a mixture of species (Scheme 1).

Spectrophotometric assays of trypsin were performed at pH 8.0 in a 100 mM HEPES buffer using Ac-Arg-pNA as the substrate (*14*). The resulting data were intrepreted using the equation for cooperative binding of two ligands, I (4-aminobutanol) and B (borate), both of which are competitive with substrate binding (*26*). This leads to a velocity equation of the form

$$\frac{v}{v_{\mathrm{mas}}} = \frac{[\mathrm{S}]}{K_{\mathrm{s}}\left(1 + \frac{[\mathrm{I}]}{K_{\mathrm{I}}} + \frac{[\mathrm{B}]}{K_{\mathrm{B}}} + \frac{[\mathrm{I}][\mathrm{B}]}{\alpha K_{\mathrm{I}} K_{\mathrm{B}}}\right) + [\mathrm{S}]} \qquad (1)$$

where S is the substrate, K_S is the Michaelis constant, and K_I and K_B are the inhibition constants corresponding to inhibitors I and B, respectively. Kinetic analysis of initial rate data as a function of the substrate and borate or 4-aminobutanol yielded the following values: $K_S = 3.47$ mM, $K_B = 80$ mM, and $K_I = 3.5$ mM. This K_S value is somewhat higher than the reported value of 1.08 mM obtained under slightly different conditions (*14*). Direct fitting of eq 1 using Mathematica yielded an α of 0.2. A value of α below 1.0 indicates positive cooperativity of the two inhibitors. Additionally, a reciprocal plot of the initial velocity as a function of borate at variable concentrations of 4-aminobutanol is predicted to yield a series of linear relationships described by (*26*)

$$\frac{1}{v_{\mathrm{i}}} = \frac{K_{\mathrm{S}}}{[\mathrm{S}]V_{\mathrm{max}}K_{\mathrm{B}}}\left(1 + \frac{[\mathrm{I}]}{\alpha K_{\mathrm{I}}}\right)[\mathrm{B}] + \frac{1}{V_{\mathrm{max}}}\left(1 + \frac{K_{\mathrm{S}}}{[\mathrm{S}]} + \frac{K_{\mathrm{S}}[\mathrm{I}]}{[\mathrm{S}]K_{\mathrm{I}}}\right) \qquad (2)$$

The corresponding Yonetani−Theorell plot (*26*, *27*) was fit using two adjustable parameters, V_{max} and α (Figure 3), and yielded a similar value (α = 0.23). The (negative) concentration at the point of convergence corresponds to $-\alpha K_B$. Although this degree of cooperativity is significant, it is considerably below the level that is observed for the cooperative inhibition of γ-GT by a serine and borate mixture. The data in Table 1 of Tate and Meister (*5*) along with some reasonable assumptions yield an α of $\sim 10^{-4}$ for the L-serine−borate complex and an α of $\sim 10^{-3}$ for the D-serine−borate complex. We propose that the lower level of synergy observed for 4-aminobutanol−borate inhibition of tryspin arises due to the lack of structural constraints near the CH_2OH end of the aminobutanol molecule, reducing the likelihood of formation of the ternary complex. However, the value obtained is clearly consistent with the NMR data indicating formation of a ternary complex.

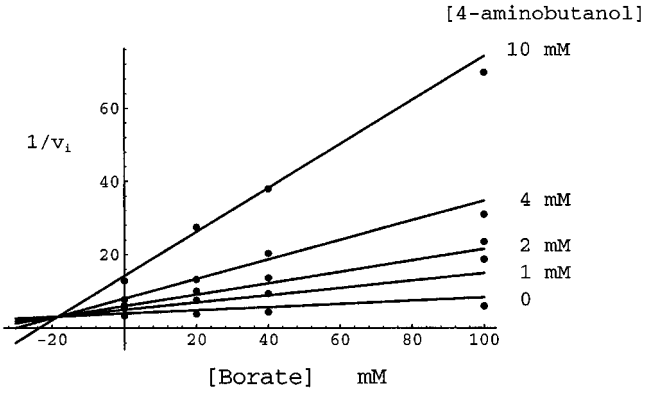


FIGURE 3: Yonetani−Theorell plot of the reciprocal initial rates as a function of borate concentration at 4-aminobutanol concentrations of 0, 1, 2, 4, and 10 mM. Studies were performed in 100 mM HEPES buffer (pH 8.0) using 200 nM porcine pancreatic trypsin and 0.3 mM Ac-Arg-pNA. Data were fit to eq 2 using a K_S of 3.47 mM, a K_B of 80 mM, and a K_I of 3.5 mM, and using V_{max} and α as adjustable parameters.

CONCLUSIONS

The results presented here provide the first direct demonstration that a stable, ternary complex is formed from a serine protease (trypsin), borate, and an alcohol. These results support the inferences discussed above for the formation of an analogous stable borate−serine−γ-GT complex, for which direct NMR or crystallographic structural evidence has been very difficult to obtain (*28*). There is no obvious constraint suggesting uniqueness of this phenomenon, so it is reasonable to anticipate that such complexes can form in many, perhaps most, enzymes of this class. As in the example of γ-GT, formation of analogous complexes may be important in understanding the biochemical and physiological actions of borate.

Ternary complex formation may involve binding of 4-aminobutanol−boric acid monoester from solution, but more probably involves ester formation at the trypsin active site. From a mechanistic standpoint, the binding of borate to His^{57} $N_{\epsilon 2}$ (*21*) and 4-aminobutanol to the S1 specificity pocket of the trypsin could provide an initial structure leading to the formation of a covalently linked complex. In some of the ligand−borate kinetic studies of Pizer and co-workers (*29*, *30*), the substitution reaction leading to biligand complexes occurs more rapidly than the addition reaction of the first ligand to the $B(OH)_3$. Thus, the hydroxyl groups of Ser^{195} and 4-aminobutanol could substitute for the hydroxyl ligands after formation of the initial complex with histidine. Nevertheless, a boronate substitution reaction does not seem to be significant for inhibition by boronic acids which appear to react only in the acid form (*31*, *32*). We also note that there have been several reports on the formation of ternary complexes formed from serine proteases with boronate inhibitors and alcohols or polyols (*33*−*35*). It has been suggested that such complexes may form the basis

for the development of more potent and specific inhibitors, although the realization of such inhbitors is apparently not straightforward (*36*, *37*). Such structures suggest the possibility of analogous doubly substituted borinic acid inhibitors (*38*) or related constructs. However, for some of these examples, no cooperative inhibition has been demonstrated, while for the complexes that involve tetrahedral boronate adducts, the ligands might be expected to compete with the enzyme for the boronate rather than to enhance inhibition. The latter conclusion is suggested by previous studies of serine protease inhibition by aryl boronic acids that show a reduction in the level of inhibition at high pH, which has been interpreted to indicate that only the acid forms are active as inhibitors (*31*, *32*).

In addition to the physiological significance of ternary complex formation by serine proteases, borate, and alcohol, the observation of such complexes has important implications for the development of tight binding ligands, as in the example of γ-GT discussed above. Thus, as noted above, the γ-GT inhibitor L-2-amino-4-boronobutanoic acid, obtained by replacing the labile serine−borate ester with a nonlabile methylene linker, exhibits potency more than 4 orders of magnitude greater than that of an L-serine/borate mixture (*7*, *8*). Thus, identification of an alcohol, ROH, capable of forming a ternary complex implies that the corresponding boronic acid, $RCH_2B(OH)_2$, will be a more potent inhibitor. Further, since the active site serine is subject to tetrahedral adduct formation with aldehydes (*39*, *40*) and fluoroketones (*41*, *42*), one can predict that RCH_2CHO and $RCH_2(C{=}O)CFR'R''$ will also be inhibitors. More generally, the ability of borate to coordinate with other types of ligands such as the ring nitrogen of histidine or carboxylate groups suggests that the use of this strategy may be subject to significant extrapolation. Further studies on related alcohol−borate−trypsin complexes are currently in progress in our lab.

REFERENCES

1. Hunt, C. D. (1998) *Biol. Trace Elem. Res. 66*, 205−225.
2. Hunt, C. D., and Idso, J. P. (1999) *J. Trace Elem. Exp. Med. 12*, 221−233.
3. Fail, P. A., Chapin, R. E., Price, C. J., and Heindel, J. J. (1998) *Reprod. Toxicol. 12*, 1−18.
4. Revel, J. P., and Ball, E. G. (1959) *J. Biol. Chem. 234*, 577−582.
5. Tate, S. S., and Meister, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4806−4809.
6. Inoue, M., Hiratkae, J., Suzuki, H., Kumagai, H., and Sakata, K. (2000) *Biochemistry 39*, 7764−7771.
7. London, R. E., and Gabel, S. A. (2001) *Arch. Biochem. Biophys. 385*, 250−258.
8. Griffith, O. W., and Meister, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 268−272.
9. Walker, B., and Lynas, J. F. (2001) *Cell. Mol. Life Sci. 58*, 596−624.
10. Kettner, C. A., and Shenvi, A. B. (1984) *J. Biol. Chem. 259*, 5106−5114.
11. Zhong, S., Haghjoo, K., Kettner, C., and Jordan, F. (1995) *J. Am. Chem. Soc. 117*, 7048−7055.
12. Plateau, P., and Gueron, M. (1982) *J. Am. Chem. Soc. 104*, 7310−7311.
13. Nishi, N., and Noguchi, J. (1973) *Bull. Chem. Soc. Jpn. 46*, 572−576.
14. Tsunematsu, H., Nishimura, H., Mizusaki, K., and Makisumi, S. (1985) *J. Biochem. 97*, 617−623.
15. Adebodun, F., and Jordan, F. (1988) *J. Am. Chem. Soc. 110*, 309−310.
16. Zhong, S., Jordan, F., Kettner, C., and Polgar, L. (1991) *J. Am. Chem. Soc. 113*, 9429−9435.
17. Tsilikounas, E., Kettner, C. A., and Bachovchin, W. W. (1993) *Biochemistry 32*, 12651−12655.
18. Baldwin, J. E., Claridge, T. D. W., Derome, A. E., Schofield, C. J., and Smith, B. D. (1991) *Biorg. Med. Chem. Lett. 1*, 9−12.
19. Coddington, J. M., and Taylor, M. J. (1989) *J. Coord. Chem. 20*, 27−38.
20. van Haveren, J., Peters, J. A., Batelaan, J. G., Kieboom, A. P. G., and van Bekkum, H. (1989) *Rec. Des. Trav. Chim. Pays-Bas 108*, 179−184.
21. Tsilikounas, E., Kettner, C. A., and Bachovchin, W. W. (1992) *Biochemistry 31*, 12839−12846.
22. Robillard, G., and Shulman, R. G. (1974) *J. Mol. Biol. 86*, 541−558.
23. House, K. L., Garber, A. R., Dunlap, R. B., and Odom, J. D. (1993) *Biochemistry 32*, 3468−3473.
24. Bao, D., Cheng, J.-T., Kettner, C., and Jordan, F. (1998) *J. Am. Chem. Soc. 120*, 3485−3489.
25. Bao, D., Huskey, W. P., Kettner, C. A., and Jordan, F. (1999) *J. Am. Chem. Soc. 121*, 4684−4689.
26. Segel, I. H. (1975) *Enzyme Kinetics*, pp 481−487, Wiley-Interscience, New York.
27. Yonetani, T., and Theorell, H. (1964) *Arch. Biochem. Biophys. 106*, 243.
28. Sakai, H., Sakabe, N., Sasaki, K., Hashimoto, W., Suzuki, H., Tachi, H., Kumagai, H., and Sakabe, K. (1996) *J. Biochem. 120*, 26−28.
29. Pizer, R., and Selzer, R. (1984) *Inorg. Chem. 23*, 3023−3026.
30. Pizer, R., and Ricatto, P. J. (1994) *Inorg. Chem. 33*, 2402−2406.
31. Philipp, M., and Bender, M. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 478−480.
32. Nakatani, H., Uehara, Y., and Hiromi, K. (1975) *J. Biochem. 78*, 611−616.
33. Suenaga, H., Mikami, M., Yamamoto, H., Harada, T., and Shinkai, S. (1995) *J. Chem. Soc., Perkin Trans. 1*, 1733−1738.
34. Katz, B. A., Finer-Moore, J., Mortezaei, R., Rich, D. H., and Stroud, R. M. (1995) *Biochemistry 34*, 8264−8280.
35. Wienand, A., Ehrhardt, C., Metternich, R., and Tapparelli, C. (1999) *Bioorg. Med. Chem. Lett. 7*, 1295−1307.
36. Tian, Z.-Q., Brown, B. B., Mack, D. P., Hutton, C. A., and Bartlett, P. A. (1997) *J. Org. Chem. 62*, 514−522.
37. Von Matt, A., Ehrhardt, C., Burhkard, P., Metternich, R., Walkinshaw, M., and Tapparelli, C. (2000) *Bioorg. Med. Chem. Lett. 8*, 2291−2303.
38. Steiner, S. J., Bien, J. T., and Smith, B. D. (1994) *Bioorg. Med. Chem. Lett. 4*, 2417−2420.
39. Lowe, G., and Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 815−817.
40. Chen, R., Gorenstein, D. G., Kennedy, W. P., Lowe, G., Nurse, D., and Schultz, R. M. (1979) *Biochemistry 18*, 921−926.
41. Imperiali, B., and Abeles, R. H. (1986) *Biochemistry 25*, 3760−3767.
42. Liang, T. C., and Abeles, R. H. (1987) *Biochemistry 26*, 7603−7608.
43. Stein, R. L., DeCicco, C., Nelson, D., and Thomas, B. (2001) *Biochemistry 40*, 5804−5811.

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