Fluorine-19 NMR Studies of Fluorobenzeneboronic Acids. 2. Kinetic Characterization of the Interaction with Subtilisin Carlsberg and Model Ligands

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Abstract: The interaction of 4-fluoro-substituted benzeneboronic acids with subtilisin Carlsberg has been studied by ^{19}F NMR. At a field strength of 8.5 T, the inhibitors 4-fluoro- and 3-chloro-4-fluorobenzeneboronic acids are in slow exchange with the enzyme and exhibit bound shifts typical of boronate species. Dissociation rate constants determined by magnetization transfer were found to be pH independent in the range from 6.4 to 7.4, with values of 156 s^{-1} (4-fluoro) and 24 s^{-1} (3-chloro-4-fluoro) at pH 7.4, 21 °C. These values are similar to previous temperature-jump determinations of the slow component of the dissociation of analogous ligands from subtilisin BPN′, demonstrating that the previously observed rates correspond to the boronate → boronic acid transition. The interaction of 4-fluorobenzeneboronic acid (FBA) with 2-pyridylcarbinol, a model ligand for the histidine and serine groups of the active site, was also studied and demonstrated to involve chelation with both the hydroxyl oxygen and pyridyl nitrogen. Nitrogen-14 NMR studies indicate that, when complexed to the boronate, the pyridyl nitrogen exhibits a shift intermediate between the values for the protonated and unprotonated forms. This result is in close agreement with ^{15}N NMR studies of boronate inhibitors bound to the active site of [ϵ-^{15}N]histidine-labeled α-lytic protease. The dissociation rate constant for the 4-fluorobenzeneboronate–pyridylcarbinol complex of 22.5 s^{-1} is considerably slower than the dissociation rate constant for the corresponding subtilisin–boronate complex. The slower dissociation kinetics for the model complex is consistent with the conclusion that such bidentate chelation is not present for the boronate–subtilisin system under study.

Introduction

Serine proteases constitute a large and functionally varied group of proteolytic enzymes, which are subject to reversible inhibition by boric and boronic acids acting as transition-state analogs.[1–3] In addition to the insight which such interactions provide into the catalytic mechanism, boronate-based inhibitors are of potential clinical interest.[4] Recent ^{15}N NMR,[5,6] ^{11}B NMR,[7] and crystallographic[8,9] studies of the complexes formed between boronate inhibitors and several enzymes from this class have demonstrated that several different binding modes can exist. While unequivocal evidence exists for the formation of tetrahedral boronate adducts involving a direct bond between the boron and the oxygen of the active-site serine,[10,11] recent studies of α-lytic protease containing ^{15}N-enriched histidine demonstrate that for some inhibitors a direct boron–nitrogen bond is formed.[5,6] Crystallographic studies show that in at least two instances bonding to both the serine oxygen and the histidine nitrogen can occur.[8b,9]

As shown in the accompanying manuscript,[12] fluorine-19 NMR studies of 4-fluoro-substituted benzeneboronic acids provide a useful and sensitive means of characterizing the chemistry at the boron atom, while exhibiting generally more favorable NMR properties from the standpoint of broadening and sensitivity. Such an approach is particularly useful for characterizing the binding kinetics, as illustrated here and in the accompanying paper. In the present studies, we have investigated the interaction of 4-fluorobenzeneboronic acids with both subtilisin Carlsberg and with several model ligands, particularly 2-pyridylcarbinol, which provides a potential model for a tetrahedral boronate complexed to both an aliphatic hydroxyl and a ring nitrogen.

Materials and Methods

Subtilisin Carlsberg derived from *Bacillus licheniformis* (MW 27 287) was obtained in crystalline form from Sigma. The 8-hydroxyquinoline-5-sulphonic acid (HQS),[13] 4-fluorobenzeneboronic acid (FBA), and 3-chloro-4-fluorobenzeneboronic acid (CFBA) were obtained from Lancaster Synthesis, Inc. The model ligands 2-, 3-, and 4-pyridylcarbinol and 4-hydroxymethylimidazole were purchased from Aldrich. Fluorine-19 NMR studies were carried out on a Nicolet NT-360 multinuclear NMR spectrometer using a 5-mm ^{1}H probe tuned to the fluorine resonance frequency of 339.7 MHz. Internal trifluoroacetate was used as a ^{19}F chemical shift standard in some of the studies.

Since ^{19}F spectra typically contained only two resonances corresponding to uncomplexed and to ligand- or enzyme-complexed species, magnetization transfer studies were carried out using the selective inversion

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(13) Abbreviations: BBA, benzeneboronic acid; CBA, 3-chlorobenzeneboronic acid; CFBA, 3-chloro-4-fluorobenzeneboronic acid; FBA, 4-fluorobenzene boronic acid; HQS, 8-hydroxyquinoline-5-sulphonic acid; NMR, nuclear magnetic resonance; 2PC, 2-pyridylcarbinol; TFA, trifluoroacetate.

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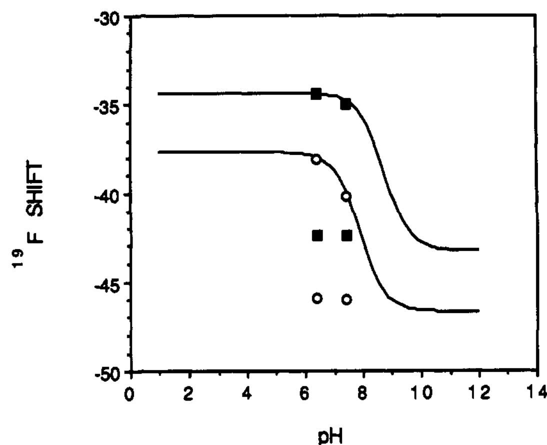

Figure 1. ^{19}F shifts in ppm of FBA (■) and CFBA (○) in the presence of subtilisin Carlsberg referenced to internal TFA. Each sample contained sufficient inhibitor to allow observation of two resonances as in Figure 4, corresponding to free and bound species. The theoretical pH titration curves for FBA and CFBA correspond to the data of Figure 1 of the preceding paper.[12] The shift of the free species in the presence of enzyme is seen to be very close to that measured in the absence of enzyme, while the shifts of the bound boronates are ~1 ppm downfield of the shift of the corresponding boronate species.

Table 1. ^{19}F Chemical Shifts of Model Complexes

boronic acid	ligand	Δ(B–F)^a (ppm)	shift^b (ppm)
FBA	subtilisin	7.59	–42.4
	OH⁻	8.54	–43.3
	catechols	6.54–6.74	–41.3 to –41.5
	carbohydrates	6.54–7.64	–41.3 to –42.4
	α-hydroxy carboxylic acids	6.34–6.44	–41.1 to –41.2
	2PC	5.38	–40.14
	HQS	4.49	–39.25
CFBA	subtilisin	6.48	–45.9
	OH⁻	7.28	–46.7
	catechols	5.15–5.35	–44.7 to –44.9
	carbohydrates	5.25–6.25	–44.8 to –45.8
	α-hydroxy carboxylic acids	4.85–4.95	–44.4 to –44.5
	2PC	3.90	–43.4
	HQS	2.71	–42.3

^{a} Shift differences between bound and uncomplexed boronic acid at pH 7.2. In a few cases, the results were calculated from data measured at pH 7.4 based on the titration curve for the uncomplexed boronic acid. ^{b} Shifts measured at 21 °C relative to TFA.

technique of Robinson et al.,[14] using the pulse sequence

$$\text{delay}-90°_x-\tau_1-90°_x-t_m-90°_\phi-\text{acq} \quad (1)$$

where $\tau_1 = 1/(2|\delta_f - \delta_b|)$, t_m is the variable delay during which transfer of magnetization occurs, and the carrier frequency of the radio frequency is set at the position of the resonance (free or bound) which is selectively inverted. The phase ϕ of the final 90° pulse and the receiver are cycled through x, y, $-x$, $-y$.

The analysis of the data was based on the approach described by Perrin and Engler[15] in which two series of experiments are carried out with the resonance of the uncomplexed species or the adduct selectively inverted. As described in the accompanying paper,[12] diagonalization of the resulting series of data matrices obtained and linear fits of the resulting elements as a function of the mixing time give the four elements of the relaxation matrix. As in the studies of kinetics with smaller ligands, rate measurements were found to be reproducible to within ±5% for repeated measurements on the same sample but to within ±15% for different samples made up separately.

Nitrogen-14 NMR spectra were obtained on an NT-360 NMR spectrometer using a 10 mm broad-band probe tuned to the ^{14}N frequency of 26.09 MHz. Internal KNO_3 was used as a chemical shift standard.

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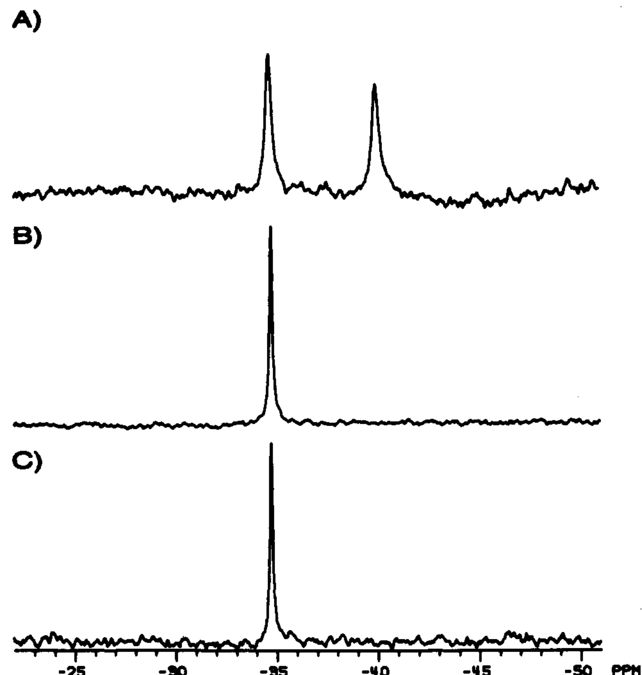

Figure 2. ^{19}F spectrum of 3 mM FBA in the presence of (A) 25 mM 2-pyridylcarbinol; (B) 3-pyridylcarbinol; (C) 4-pyridylcarbinol. Samples contained 50 mM phosphate, pH 7.4. Chemical shifts are relative to external trifluoroacetate.

Results

Chemical Shift Data. As discussed in the preceding paper,[12] the ^{19}F resonances of FBA and CFBA undergo a chemical shift of ~9 ppm as a function of pH, which corresponds to the transition from boronic acid to boronate species. The pK values of 8.7 and 7.9 are close to the values for benzeneboronic acid[16] and *m*-chlorobenzeneboronic acid,[17] reflecting the small para Hammett constant of fluorine. The influence of ring substituents on the inhibition constants K_I has been subject to a number of studies,[2,3,18,19] with the general result that enzyme inhibition is correlated with electron-withdrawing potency of the substituents.

The ^{19}F NMR spectra of FBA or CFBA in the presence of subtilisin exhibit two resonances, indicating slow exchange between the uncomplexed and enzyme-complexed species on the fluorine shift time scale. The ^{19}F shifts (referenced to internal TFA) of the FBA and CFBA resonances observed in the presence of enzyme are shown in Figure 1, along with the pH titration curves for both species determined in the absence of enzyme. As is apparent from this figure, the shifts of the free species are very close, $\Delta\delta < 0.2$ ppm, to the values determined in the absence of enzyme, while the shifts of the bound species are slightly downfield of the corresponding boronate shifts. For the bound species, measured chemical shift differences are 0.9 ppm for Δ[δ(FBA–subtilisin) – δ(FBOH⁻)] and 0.8 ppm for Δ[δ(CFBA–subtilisin) – δ(CFBOH⁻)] (Table 1).

Since recent ^{15}N NMR[5,6] and crystallographic[8b,9] evidence indicates that the histidine of the catalytic triad can be directly involved in the binding of boronic acid inhibitors, the interaction of FBA with several model ligands containing both hydroxyl and ring nitrogen groups was studied. The ^{19}F NMR spectrum of FBA in the presence of 2-pyridylcarbinol exhibits two resonances with a chemical shift difference of 5.6 ppm at pH 7.4 (Figure 2).

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The significance of the boron–nitrogen bond for the interaction is clearly supported by the observation that separate fluorine resonances are not observed for FBA in the presence of 3-pyridylcarbinol or 4-pyridylcarbinol (Figure 2). The shift difference between FBA and the FBA–2PC complex is smaller than the corresponding difference between the free and subtilisin-complexed FBA of 7.5 ppm measured at the same pH (7.4). It is also smaller than the ^{19}F shift differences observed with any of the bidentate oxygen ligands examined in the accompanying study, which include catechols, carbohydrates, and α-hydroxy carboxylic acids (Table 1 and Table 1 of ref 12). Similar conclusions hold for CFBA (Table 1); i.e., complexation with 2PC resulted in a smaller shift than complexation of OH^-, subtilisin, or any of the other bidentate ligands tested.

The binding of FBA to another mixed oxygen, nitrogen bidentate ligand, 8-hydroxyquinoline-5-sulphonate (HQS) was strong enough to result in separate bound and free resonances at pH 7.2, although there is substantial exchange broadening. The interaction of FBA with HQS at this pH corresponds to an apparent dissociation constant of ~250 mM. As in the case of the complex formed with 2PC discussed above, the observed shift difference between the uncomplexed FBA and the 2PC complex of 4.5 ppm determined at pH 7.2 is significantly smaller than the values observed with any of the bidentate oxygen ligands (Table 1). Similarly, the CFBA–HQS complex gives a smaller upfield shift than the other CFBA complexes studied (Table 1).

Analogous ^{19}F NMR studies of FBA in the presence of 4-(hydroxylmethyl)imidazole did not exhibit separate resonances for free and bound species. However, the FBA fluorine resonance does exhibit a shift dependent on the concentration of the 4-(hydroxymethyl)imidazole. Analysis of the observed FBA shift as a function of (hydroxymethyl)imidazole concentration yields an apparent dissociation constant of 0.4 M at pH 8.0. A corresponding pH-imdependent dissociations constant can be determined, as described by Koehler et al.[20] and Nakatani and Hiromi,[19b] corresponding to the interaction of benzeneboronic acid with imidazole, according to the relation

$$K_D^\circ = \frac{K_D^{app}}{(1 + e^{pK_L - pH})(1 + e^{pH - pK_B})} \qquad (2)$$

where pK_L and pK_B correspond to the pK values of the ligand and the boronic acid, respectively. Using a $pK_L = 7.3$ for 4-(hydroxymethyl)imidazole and 8.7 for FBA, the $K_D^{app} = 0.4$ M at pH 8.0 corresponds to $K_D^\circ = 0.18$ M. This result corresponds to a stability constant of 5.6 M^{-1}, similar to the value of 10.7 M^{-1} obtained by Koehler et al.[20] for imidazole–benzeneboronic acid. The fast exchange kinetics and K_D° value obtained therefore support the conclusion that the interaction is exclusively with the imidazole nitrogen. Since the studies of Koehler et al.[20] demonstrate that imidazole binds to BBA more strongly than pyridine, the stronger binding of FBA to 2PC compared with 4-(hydroxymethyl)imidazole must reflect a geometric constraint of the latter which is unfavorable for chelation.

Nitrogen Shifts. Since N-15 chemical shift data have been used diagnostically for the presence of a boron–nitrogen bond in α-lytic protease,[5,6] the effect of boron complexation on the nitrogen chemical shift of 2-pyridylcarbinol was also investigated. Due to sensitivity considerations, the 99.6% abundant ^{14}N isotope was studied rather than ^{15}N, but the shifts in ppm will be identical. The ^{14}N resonance of 2-pyridylcarbinol gave a 93 ppm titration shift with a pK of 5.0, as expected for a pyridine derivative (Figure 3). A spectrum obtained on a sample containing 100 mM PC plus 75 mM FBA at pH 7.2 showed two resonances corresponding to the (unprotonated) PC and to the FBA–PC complex. As shown in Figure 3, the ^{14}N resonance of the complex was roughly midway

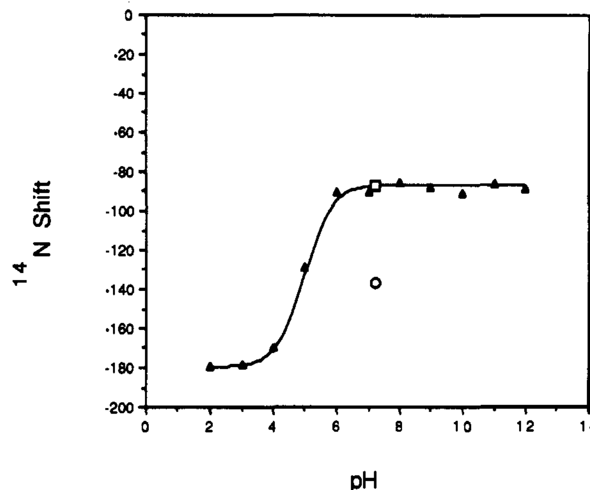

Figure 3. Nitrogen-14 shifts of 100 mM 2-pyridylcarbinol as a function of pH (▲). Also shown are the chemical shift positions of the two ^{14}N resonances observed in a solution containing 100 mM 2-pyridylcarbinol and 75 mM FBA at pH 7.2, corresponding to uncomplexed PC (□) and to the FBA–PC complex (○). Chemical shifts are relative to the ^{14}N signal of internal KNO_3.

in shift between the protonated and unprotonated forms of PC. This result is in excellent agreement with the data on imidazole and the imidazole–BBA complex reported by Bachovchin et al.,[5] in which the ^{15}N shift of the complex is close to the midpoint of the shifts of the protonated and unprotonated nitrogen of the imidazole. This agreement supports the conclusion that the PC provides a good model for the imidazole–boronate interaction in α-lytic protease and presumably in other serine proteases such as porcine pancreatic elastase which have been shown to exhibit a corresponding interaction with some boronate inhibitors.

Kinetic measurements. The slow kinetic behavior corresponding to the two resonances observed for FBA or CFBA in the presence of subtilisin was studied using magnetization transfer methods. As discussed in Materials and Methods, each of the two resonances is sequentially inverted and spectra are subsequently obtained at regular intervals (Figure 4). The resulting two rate constants are related by the equilibrium condition

$$k_{BF}/k_{FB} = p_B/p_F \qquad (3)$$

where p_F and p_B are the fractions of uncomplexed and complexed inhibitor and k_{BF} and k_{FB} the inverse lifetimes for the bound and free states, respectively. For a simple model of enzyme inhibitor complexation described by the relation

$$I + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}I \qquad (4)$$

$k_{BF} = k_{-1}$ and $k_{FB} = [E]k_{-1}$. Since there is evidence that the actual complexation of boronate inhibitors with enzyme is a multistep process,[19a,c] the "free" and "bound" states are defined from the standpoint of the NMR kinetic experiments by the chemical shift. Thus, the "free" state corresponds to all physical states which are in fast exchange with the uncomplexed inhibitor. Dissociation rate constants obtained using this approach for FBA- and CFBA–enzyme complexes are summarized in Table 2. In addition, analogous data obtained for the complexes with 2-pyridylcarbinol are also summarized. From these data, it can be seen that (1) the addition of the meta chloro substituent reduces the dissociation rate constants by a factor of ~6–8 and (2) there appeared to be a small but measurable effect of the phosphate buffer on the FBA dissociation rate but not on the CFBA dissociation rate.

For the purpose of comparison, the dissociation rates corresponding to the slow kinetic step, k_{-2}, observed by Nakatani et

(20) Koehler, K. A.; Jackson, R. C.; Lienhard, G. E. *J. Org. Chem.* **1972**, *37*, 2232–2237.

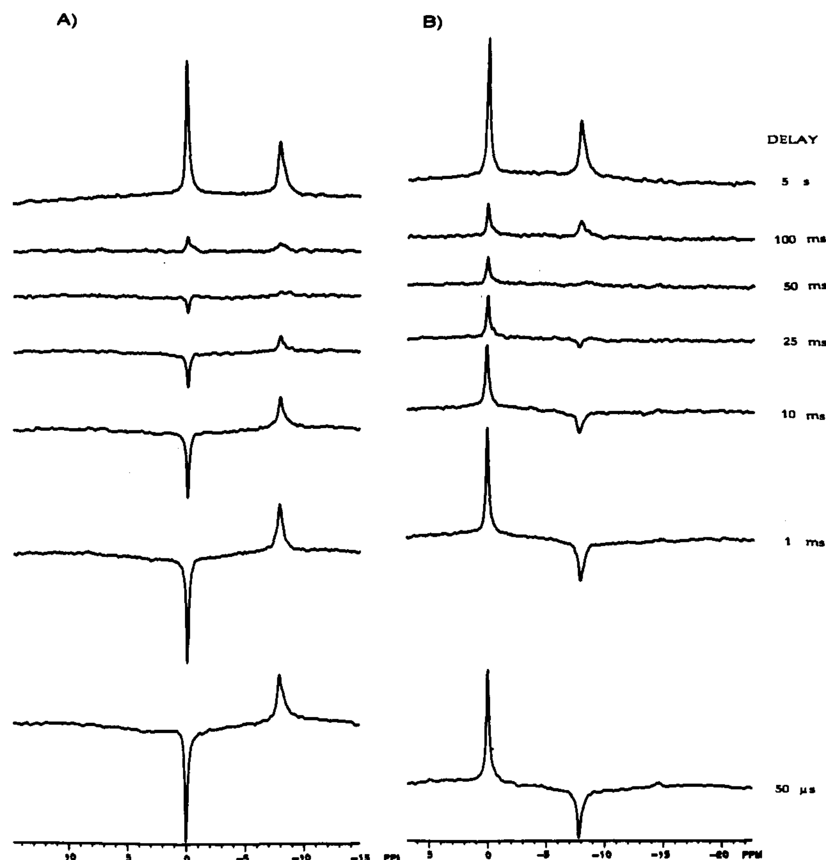

Figure 4. [19]F selective inversion recovery experiment of 4 mM CFBA in the presence of 2.5 mM subtilisin Carlsberg. No internal standards were added for the magnetization transfer measurements, and the shift of the uncomplexed FBA was arbitrarily set at 0. The measurement involves studies in which either the free or the bound peak is selectively inverted. Other conditions were T = 21 °C, pH = 6.4, 4096 data points, sweep width = 10 kHz, number of acquisitions per spectrum = 800.

Table 2. Dissociation Rate Constants

boronic acid	ligand	pH	k_{-1}[a] (s^{-1}) solv 1	k_{-1}[a] (s^{-1}) solv 2	k_{-2}[b] (s^{-1})
FBA	subtilisin	6.4	200	170	
BBA		6.5			260
FBA		7.4	200	160	
				160[c]	
CFBA		6.4	27	26	
CFBA		7.4	24	24	
CBA		6.5			39
FBA[d]	PC	8.4	180		
FBA		7.4	190	23	
FBA		6.4	150	26	
FBA		5.4	130	30	
CFBA[d]	PC	7.4		23	
CFBA		6.4		28	

[a] Dissociation rates from subtilisin determined at 21 °C in 50 mM phosphate (solvent 1) or 0.1 M KCl (solvent 2). [b] Slow dissociation rate constants k_{-2} for benzeneboronic acid (BBA) or 3-chlorobenzeneboronic acid (CBA) from subtilisin BPN′ determined at 15 °C, pH 6.5 (Nakatani et al.[19c]). [c] Measured in 0.1 M KCl + 0.1 M phosphate. [d] Concentrations of FBA or CFBA were 6 mM. PC concentration adjusted to give similar bound and free resonance intensities.

al.[19c] in stopped-flow kinetic studies of the interaction of benzeneboronic acid (BBA) and *m*-chlorobenzeneboronic acid (CBA) with subtilisin BPN′ at 15 °C are also included in the table. Since the para Hammett constant of fluorine is relatively small[21] (0.06), the effects of the fluorine on the boron interactions would presumably be minimal. Given the differences in systems and conditions, the agreement between the two types of measurement is very reasonable.

If the complexation reaction is assumed to represent a single step and the inhibition constants K_I = 0.69 mM for BBA[22] and ~0.1 mM for CFBA (estimated from Figure 2 of ref 2) are assumed valid for the fluorinated analogs and set equal to the dissociation constants, association rate constants of 2.3×10^5 and 2.4×10^5 M^{-1} s^{-1} are obtained for the two inhibitors. These results are more than 2 orders of magnitude below values of 10^8–10^9 M^{-1} s^{-1}, which would be expected for diffusion controlled reactions. Hence, the interaction of the inhibitors with the enzyme which is associated with the observation of the chemically shifted resonance is not diffusion controlled.

Kinetic studies of the FBA and CFBA complexes with 2-pyridylcarbinol were also carried out to determine whether this might be a reasonable model for enzyme binding from a kinetic standpoint. Although the results for the FBA complex obtained in the 50 mM phosphate buffer are in fairly close quantitative agreement with the results for the enzyme, this agreement disappears when the measurements are carried out in 0.1 M KCl (Table 1). Thus, in contrast to the results for the enzyme, there is a large effect of the phosphate buffer on the dissociation kinetics of the FBA–PC complex. This result is consistent with observations on a range of bidentate oxygen ligands which indicate a signicicant buffer-catalyzed enhancement of the binding kinetics.[12] Hence, the dissociation kinetics of the FBA–2PC system are considerably slower than those for FBA–subtilisin. In contrast, the dissociation rate constant for CFBA–2PC was similar to the measurement for the CFBA–subtilisin complex (Table 2).

Apparent equilibrium dissociation constants of 26 and 8.5 mM for the FBA–2PC and CFBA–2PC complexes were determined at pH 7.4, 21 °C. On the basis of the observed dissociation rate constants, these correspond to association rate constants k_1 = 8.7

(21) Perrin, D. D.; Dempsey, B.; Serjeant, E. P. *pK$_a$ Predictions for Organic Acids and Bases*; Chapman and Hall: London, 1981.

(22) Lindquist, R. N.; Terry, C. *Arch. Biochem. Biophys.* **1974**, *160*, 135–144.

$\times 10^2$ and 2.7×10^3 M^{-1} s^{-1} for the two boronates. As with the enzyme, these are well below a diffusion-controlled limit and are also much smaller than the association rate constants calculated above for binding to the enzyme.

Discussion

The presence of a fluorine nucleus in 4-fluorobenzeneboronic acid provides an NMR probe which is both sensitive to the hybridization of the boron and sensitive from the standpoint of NMR detection. This probe is an ideal complement to the ^{11}B NMR studies of benzeneboronic acids, which is particularly useful for kinetic characterization, as described here. This reflects in particular the large fluorine chemical shift range which allows magnetization transfer studies to be utilized for rate determinations, as well as the advantages of working with a spin $^1/_2$ nucleus. Since the observation of magnetization transfer requires that the exchange rate exceed or at least be comparable to $1/T_1$, this type of measurement will presumably be more difficult or impossible using boron-11 due to the more rapid quadrupolar relaxation. Preliminary ^{11}B NMR measurements on FBA at pH 7.4 indicate a T_1 value of 1 ms, much shorter than the exchange lifetimes involved in these studies. The need for such kinetic data is also becoming increasingly apparent for the analysis of ligand–enzyme exchange data in transferred NOE studies.[23] Another advantage is the freedom from spurious signals due to boric acid contaminants which are apparently present in boronic acid preparations.[7c,d,e]

The results obtained here indicating that the shifts of subtilisin-complexed FBA and CFBA are consistent with a boronate structure are qualitatively analogous to conclusions based on recent ^{11}B NMR studies of boronic acid inhibitors of chymotrypsin and subtilisin;[7e] however, there are some significant quantitative differences. In the reported ^{11}B NMR studies, the resonances of tightly bound inhibitors show a large upfield shift consistent with the formation of a tetrahedral boronate adduct; however, there are large shift differences of both the "free" and bound resonances relative to the boronic acid and boronate values. For the case of BBA in the presence of chymotrypsin,[7e] the "free" resonance is shifted significantly upfield relative to its position in the absence of enzyme, and the bound resonance, observable at low temperature, is 11.6 ppm upfield of the resonance for benzeneboronate. This difference is greater than can be readily explained on the basis of contributions of nearby aromatic or charged residues. The ^{11}B NMR shift of the "free" species was interpreted in terms of fast exchange between the uncomplexed boronic acid and a weakly bound state, such that an averaged shift was observed.

Jordan and co-workers[7e] also reported ^{11}B NMR studies of another benzeneboronic acid inhibitor, 3-(dansylamido)phenylboronic acid in the presence of subtilisin Carlsberg, which revealed two resonances with shifts of 3.9 and −20.1 ppm. Analogous to the results for chymotrypsin, the latter value is 8.1 ppm upfield of the resonance for the corresponding free boronate species.[7e] As in the case of chymotrypsin, this contrasts with the results of the ^{19}F NMR studies of the fluorinated benzeneboronic acids, in which the shifts corresponding to the adduct are within 1 ppm of the shifts of the corresponding boronate species (Table 1).

The ^{19}F shifts of the 4-fluorobenzeneboronate adducts formed with various ligands are presumably sensitive to the chemical nature and the geometric constraints of the ligands. On the basis of the data obtained here and in the preceding paper,[12] we find that the ^{19}F shift of the $FBOH^-$ adduct is furthest upfield at −43.3 ppm relative to TFA; adducts formed with bidentate oxygen ligands with a wide variation of geometric constraints fall into the range from −41.1 to −42.5 ppm, while the adducts formed from mixed pyridyl nitrogen and hydroxyl ligands show smaller shifts (Table 1). Recent theoretical calculations suggest that C–F bond polarization resulting from electric fields produced by nearby charges may play an important role in determining ^{19}F shifts.[24] The smaller shifts observed in the nominally neutral complexes of FBA and CFBA with 2PC and HQS compared with the negatively charged boronate complexes (Table 1) may reflect this difference in net charge. Thus, to the extent that chemical shift is diagnostic, the ^{19}F shift data for the FBA and CFBA subtilisin adducts are more consistent with a model in which the bound, tetrahedral boronate species has three oxygen ligands rather than a nitrogen and two oxygen ligands. This conclusion is consistent with recent proton NMR studies of the BBA adduct with subtilisin Carlsberg, in which two downfield shifted proton resonances attributed to the active site histidine have been observed,[25] ruling out the formation of a boron–nitrogen bond. Of course, the proximity of the fluorine to other amino acid residues can result in significant contributions to the observed shift, so that reasoning from shift data alone is inconclusive. The similarity of the ^{19}F shift observed for the enzyme–FBA complex to those of the model boronate complexes could reflect the relatively weak binding of these inhibitors, so that chemical shift contributions of other protein residues which may be close to the fluorine probe tend to be averaged out due to conformational mobility of the adducts formed.

In the ^{19}F NMR studies reported here, neither the shifts nor the dissociation rate constants for FBA and CFBA varied significantly between pH 6.4 and 7.4. The slow dissociation step measured here is in fairly close quantitative agreement with the values for the slow dissociation step, k_{-2}, measured previously by temperature-jump/stopped-flow methods for the adducts formed from the nonfluorinated boronic acid analogs and subtilisin BPN′.[19a,c] However, in contrast with present results, the reported k_{-2} values exhibited a significant pH dependence in this range. These differences may reflect differences between the enzyme systems studied. The constancy of the ^{19}F shift and kinetic data is, however, consistent with recent ^1H NMR studies of House et al.,[25] indicating that the proton resonances of the active-site histidine in the adduct formed by BBA and subtilisin Carlsberg are essentially pH independent between pH 5 and 11.

Kinetic studies also indicate some differences between the model complexes and the enzyme adduct. Although the dissociation rate constant for the FBA–PC complex measured in phosphate approximates that of the enzyme, this agreement is an accidental consequence of buffer catalysis, as discussed in the accompanying paper.[12] The dissociation rate in 0.1 M KCl is substantially slower than the rate for the FBA–subtilisin complex, suggesting that a monodentate model may be more appropriate for this system. Further, as demonstrated by Koehler et al.,[20] the binding of benzeneboronic acid to imidazole is somewhat stronger than the binding to pyridine. This difference would be expected to further increase the significance of the discrepancy in rate constants between the enzyme and the model ligand. In contrast, the measured dissociation rate constant for the CFBA–PC complex was close to the dissociation rate for the enzyme adduct (Table 2). As is apparent from the data in Table 2, this agreement reflects the fact that CFBA dissociates more slowly from the enzyme than FBA, while the dissociation rate constants for CFBA–PC and FBA–PC are similar. One possible explanation for this discrepancy is the possibility of associative equilibria, as described in the accompanying paper.[12]

Currently available data for complexation of serine proteases with boronate inhibitors can be considered within the context of the model shown in Figure 5. The central features of this model are as follows: (1) a rapid, perhaps diffusion-controlled initial binding step, prior to formation of covalent bonds to the boron; (2) a second step involving the formation of a bond to the active-

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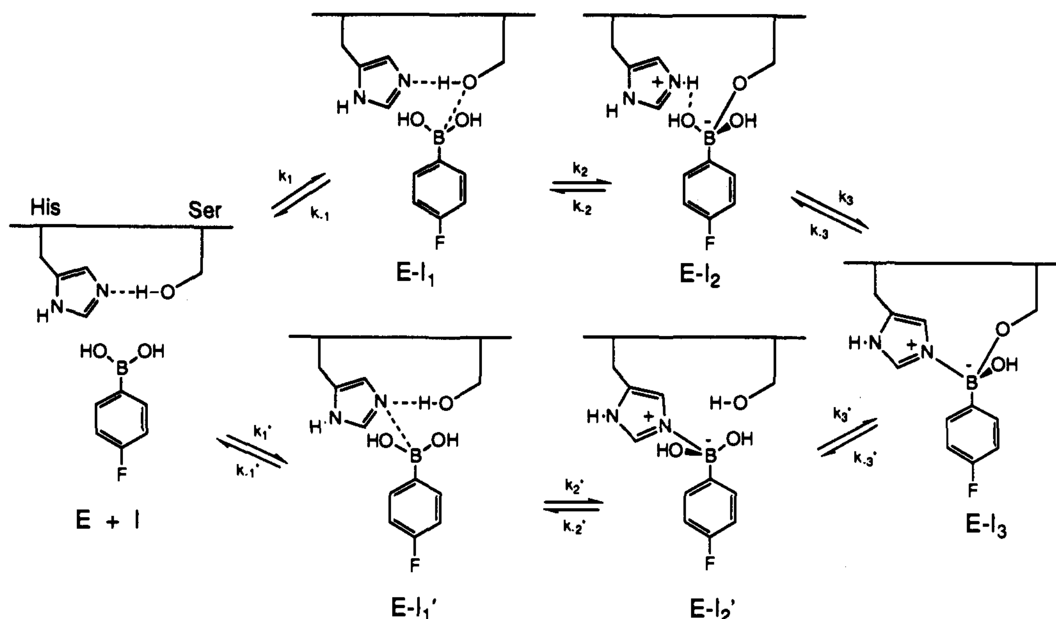
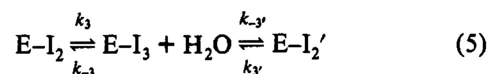


Figure 5. Interaction of FBA with the active-site residues of subtilisin. The boronic acid remains trigonal in complexes $E-I_1$ and $E-I_{1'}$. The formation of $E-I_3$ from $E-I_2$ or $E-I_{2'}$ is accompanied by loss of a water molecule which is not shown.

site serine oxygen ($E-I_2$) or histidine nitrogen ($E-I_2'$) and change in hybridization to yield a boronate–enzyme complex; (3) possibly a third step as outlined, for example, by Takahashi et al.[9] in which water is eliminated and a second covalent bond is formed to yield $E-I_3$. Evidence for the fast, initial complexation step has been obtained, for example, using stopped flow measurements.[19a,c]

Since the boron hybridization has not changed in forming the $E-I_1$ or $E-I_1'$ complexes and since dissociation from such noncovalently bound states is likely to be fast on the NMR time scale, the effects of such complexation will be to alter the observed chemical shift of the "free", i.e. not slowly exchanging, resonance. However, as shown in Figure 1, the shift of the ^{19}F resonance corresponding to the free inhibitor is essentially identical with that of uncomplexed FBA or CFBA studied at the same pH in the absence of the enzyme. In principle, this observation indicates that the fractional concentration of states $E-I_1$ or $E-I_1'$ is negligibly small and/or the shift corresponding to such species is very close to the value for free boronic acid. However, the latter alternative is ruled out on the basis of the observed shift for the CFBA. At pH 7.4, the CFBA in solution is approximately 25% in the boronate form; however, the adduct corresponding to $E-I_1$ or $E-I_1'$ involves a trigonal, boronic acid species. Hence, the equivalence of the observed shifts of the "free" resonance in samples containing enzyme or lacking enzyme indicates that the population of states with bound, trigonal boronic acid is very low.

The ^{19}F resonance of the bound boronate species may correspond to a single species, such as $E-I_2$, or to a mixture of boronate species if these interconvert with sufficient rates relative to the shift differences:



$$E-I_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E-I_3 + H_2O \underset{k_{3'}}{\overset{k_{-3'}}{\rightleftharpoons}} E-I_2' \quad (5)$$

At present, there is strong crystallographic evidence obtained on a variety of boronate inhibitors to support the involvement of the serine hydroxyl group in a direct oxygen–boron covalent bond.[8–11] Recent ^{11}B NMR studies also demonstrate that anhydroSer195 chymotrypsin does not show any resonances corresponding to bound, tetrahedral boronate inhibitors.[7e] Although recent ^{15}N NMR studies of α-lytic protease[5] indicate that species of the form $E-I_2'$ or $E-I_3$ may form, the recent observation of two downfield-shifted proton resonances (15.0 and 17.6 ppm) assigned to the active-site histidine in the BBA–subtilisin Carlsberg adduct appears to rule out such species in this system.[25] The presence of a hydrogen bond between the serine hydroxyl proton and the histidine ring nitrogen in the uncomplexed enzyme as illustrated in Figure 5 and demonstrated by ^{15}N NMR in the case of α-lytic protease[26] would tend to block the direct formation of adducts such as $E-I_{2'}$, which require the boronic acid to interact with the free ring nitrogen. Alternatively, the $E-I_{2'}$ adduct can form via the pathway illustrated by eq 5 above, which may represent a significant dissociative pathway for boronate inhibitors.

The present results demonstrate the utility of ^{19}F NMR for characterizing the nature of the interaction of fluorobenzeneboronic acids with serine proteases. The ^{19}F and ^{14}N NMR data obtained for both 4-fluorobenzeneboronic acids in the presence of 2-pyridylcarbinol and 8-hydroxyquinoline-5-sulphonate demonstrate that bidentate ligation of the boron by mixed oxygen and ring nitrogen ligands will form if not specifically precluded by geometric constraints. As data for complexes with other members of this class of enzymes become available, it may be possible to strengthen the conclusions which can be derived from chemical shift and kinetic data in terms of characterizing the structure of the enzyme–boronate complex.

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