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Ternary borate-nucleoside complex stabilization by Ribonuclease A demonstrates phosphate mimicry

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Abstract

Phosphate esters play a central role in cellular energetics, biochemical activation, signal transduction and conformational switching. The structural homology of the borate anion with phosphate, combined with its ability to spontaneously esterify hydroxyl groups, suggested that phosphate-ester recognition sites on proteins might exhibit significant affinity for non-enzymatically formed borate esters. ^{11}B NMR studies and activity measurements on ribonuclease A in the presence of borate and several cytidine analogs demonstrate the formation of a stable ternary RNase A•3'-deoxycytidine-2'-borate ternary complex that mimics the complex formed between RNase A and a 2'-cytidine monophosphate (2'-CMP) inhibitor. Alternatively, no slowly exchanging borate resonance is observed for a ternary RNase A, borate, 2'-deoxycytidine mixture, demonstrating the critical importance of the 2'-hydroxyl group for complex formation. Titration of the ternary complex with 2'-CMP shows that it can displace the bound borate ester with a binding constant that is close to the reported inhibition constant of RNase A by 2'CMP. RNase A binding of a cyclic cytidine-2',3'-borate ester, which is a structural homolog of the cytidine-2',3'-cyclic phosphate substrate, could also be demonstrated. The apparent dissociation constant for the cytidine-2',3'-borate•RNase A complex is 0.8 mM, which compares with a Michaelis constant of 11 mM for cCMP at pH 7, indicating considerably stronger binding. However, the value is 1000-fold larger than the reported dissociation constant of the RNase A complex with uridine-vanadate. These results are consistent with recent reports suggesting that *in situ* formation of borate esters that mimic the corresponding phosphate esters support enzyme catalysis.

Introduction

Phosphorylation reactions play a central role in cellular energetics, biochemical activation, signal transduction, conformational switching, and as mediators of a host of protein and peptide interactions. Thus, mimicry of biological phosphorylation has significant implications for understanding enzyme function and for the perturbation of normal phosphorylation signals. The most extensively studied phosphate mimics are oxyanions, particularly arsenate, vanadate, molybdate and tungstate [1-13]. Arsenate forms labile ADP-arsenate complexes that can compromise cellular energy production and utilization [1-3], while vanadate, bound in a trigonal bipyrimidal, pentacoordinate form, mimics phosphate transition state chemistry [4-12]. The enzyme complexes formed from these oxyanions have provided extremely useful probes for the investigation of enzyme mechanisms and also serve as transition state inhibitors of specific transphosphorylation reactions [6,9,10,13]. A second type of phosphate mimicry previously identified involves metal fluorides, which can complex with ADP/GDP and simulate ATP and GTP-dependent enzyme activation/inhibition [14-19]. Although the borate

anion adopts a structurally analogous tetrahedral geometry, and forms complexes with some phosphorylated molecules such as NAD [20,21] and ADP [22], there is little if any data supporting the conclusion that borate can in any way mimic the phosphate group. However, the lability of the borate-oxygen bond facilitates borate ester formation, and these structures could in principle be stabilized by interaction with the appropriate phosphate ester binding sites. Chemical evidence for borate mimicry of a phosphate ester has recently been reported by Sugiyama et al., who found that the enzyme L-rhamnulose-1-phosphate aldolase, a dihydroxyacetone phosphate-dependent aldolase, can utilize non-phosphorylated dihydroxyacetone in a borate buffer [23].

Recent studies of the membrane-associated γ -glutamyl transpeptidase (γ GT) have demonstrated that the enzyme can act as a template to stabilize a ternary γ GT-L-serine-borate complex at the active site, and this complex serves as a blueprint for the synthesis of relatively potent and specific isostructural boronate inhibitor: L-2-amino-4-boronobutanoic acid [24, 25]. The successful construction of a useful γ GT inhibitor based on the ternary γ GT-borate-serine complex supports the value of a more general exploration of ternary complex formation. Analogous ternary complex formation from the enzyme trypsin, borate and alcohols selected to bind to the S1 specificity pocket has been demonstrated by NMR spectroscopy and X-ray crystallography [26-28]. In these complexes, borate is covalently linked to the S1-binding alcohol and to the O γ of the active site serine (or, in the case of γ GT, the active site threonine residue). Formation/stabilization of the ester bond between the borate and the alcohol function on the ligand is not directly related to the active site chemistry, and thus is in principle possible for other classes of proteins. The present studies were designed to determine whether analogous enzyme-stabilized borate esters can form at phosphate binding sites. In order to explore this possibility, we have utilized ^{11}B NMR to investigate potential ternary complex formation of borate-nucleoside-ribonuclease A mixtures. Demonstration of such a complex has broad implications for the design of targeted ligands, as well as for borate mimicry of phosphorylation.

Methods

Type I-A Ribonuclease A derived from bovine pancreas, as well as other chemicals used in the studies, were obtained from Sigma and used without further purification. 3'-deoxycytidine was obtained from Berry & Associates (Dexter, MI). ^{11}B spectra were obtained on a Varian INOVA 500 NMR spectrometer using a Nalorac 5 mm variable temperature broadband probe tuned to 160.6 MHz and modified to reduce the ^{11}B background by replacement of the glass insert. Additionally, quartz NMR tubes (Wilma LabGlass, Buena, NJ) were used to reduce the ^{11}B background signal. ^{11}B resonances were referenced to an external solution of boric acid at pH 4.0.

NMR data were processed and analyzed using VNMR software. For the ^{11}B NMR spectra, the FID was apodized using an exponential multiplication corresponding to 5 Hz line broadening. In some cases, the spectra were characterized by a sloping baseline; this was corrected using the Varian "dc" linear baseline correction function.

For the kinetic studies, ^{31}P resonances were observed using a 5 mm Varian broadband probe tuned to 202.6 MHz. RNase A activity was monitored at 25 °C by measuring the time-dependent disappearance of the ^{31}P resonance arising from cytidine-2',3'-cyclicphosphate (cCMP) and the parallel appearance of the cytidine-3'-phosphate resonance. Samples contained 10 mM cCMP and 1 μM RNase A in 100 mM HEPES pH 8.0 buffer, as well as other test molecules as indicated. Inhibition constants for borate and 3'-deoxycytidine were obtained by fitting the data to the expression:

$$\frac{v_i}{V_{max}} = \frac{[S]}{K_s \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad [1]$$

where v_i is the initial velocity, $[S]$ and $[I]$ the substrate and inhibitor concentrations, respectively, K_s the Michaelis constant, and V_{max} and K_i were adjustable parameters. Data were fit using the non-linear fitting routine of Mathematica (Wolfram Research, Champaign, IL).

Results

Ribonuclease A cleaves single stranded RNA at the P-O5' bond in two steps, leaving a terminal pyrimidine-3'-phosphate [29]. It is inhibited by both 2'-CMP and 3'-CMP [30], while the intermediate cytidine-2',3'-cyclicphosphate (cCMP) can serve as a substrate and has been used for the spectrophotometric assay of RNase A activity [31]. A series of ^{11}B NMR studies was designed to test for ternary complex formation based on the structures of known substrate and inhibitor nucleotides. The ^{11}B NMR spectrum obtained at 5 °C on a sample containing 5 mM borate, 2 mM ribonuclease A in 50 mM HEPES pH 8.65 buffer shows a broad resonance at -5.2 ppm that is assigned to free boric acid/borate (Figure 1a). Addition of 10 mM 3'-deoxycytidine to this sample to create a ternary mixture resulted in the appearance of an additional ^{11}B resonance at $\delta = -17.54$ ppm in slow exchange with the free borate (Figure 1c). For this species, a tetrahedral geometry is inferred from the shift [e.g. 26,32]. As an important control, no additional ^{11}B resonance was observed at this position for a binary mixture of borate with 3'-deoxycytidine (Figure 1b). In combination, these results indicate the formation of a ternary complex due to stabilization of a borate ester with the 2' hydroxyl oxygen of the 3'-deoxycytidine in the presence of ribonuclease A. Apparently, the position of the 2'-hydroxyl is ideally suited for formation of an ester bond with the weakly complexed borate anion, while the 3'-hydroxyl group of 2'-deoxycytidine is not positioned in sufficient proximity to a borate oxygen to support formation of a stable ternary complex.

In contrast with the above results the ^{11}B spectrum obtained for a ternary mixture of RNase A, borate and 2'-deoxycytidine did not reveal an additional, upfield resonance (Figure 2). This result indicates that the labile borate esters formed with either the 3'- or 5'-hydroxyl groups of the 2'-deoxycytidine are not significantly stabilized by interaction with the enzyme.

It is also apparent from Figs. 1 and 2 that the ^{11}B linewidths of free boric acid/borate in samples containing RNase A is somewhat larger (approximately 600 Hz) than the linewidth in samples lacking enzyme (approximately 550 Hz). We believe that this effect most likely arises due to weak binding to other functional groups on the enzyme surface. For example, the enzyme has multiple solvent-exposed tyrosine, serine and threonine residues that can form weak, monoborate esters. If the lifetimes of these complexes are sufficient to allow the bound borate to experience the rotational correlation time of the enzyme, a significant increase in linewidth is predicted. Additionally, the borate resonance is subject to substantial, pH dependent exchange broadening due to the $\text{B}(\text{OH})_3/\text{B}(\text{OH})_4^-$ interconversion or to intermediate exchange with enzyme monoborate esters.

Thermal stability and pH dependence of 3'-deoxycytidine-2'-Borate•RNase A

The ternary RNase A•3'-deoxycytidine-2'-borate complex was further characterized by the temperature- and pH-dependence of the slowly exchanging borate resonance. The series of the spectra shown in Figure 3 was obtained under conditions similar to those used for Figure 1c except for the larger, 60 mM concentration of 3'-deoxycytidine. The resonance at $\delta = -17.5$

ppm broadens and loses intensity as the temperature is increased (Figure 3), consistent with thermal destabilization of the complex. In contrast, the linewidth of the uncomplexed $B(OH)_3/B(OH)_4^-$ species decreases as the temperature is raised, due to more rapid exchange between the acid and anion forms, as well as the shorter rotational correlation times that accompany the temperature increase. Even if the exchange contribution is eliminated, the trigonal $B(OH)_3$ species that predominates at low pH will be characterized by a greater linewidth than the $B(OH)_4^-$ species due to the effect of the lower symmetry/larger electric field gradient on quadrupolar relaxation of the ^{11}B nucleus ($s = 3/2$).

The concentration of the ternary complex at each pH value was determined based on the intensity of the corresponding ^{11}B resonance, and this value was used to determine the fraction of RNase A in the complex. At pH 5.0, the -17.5 ppm resonance becomes undetectable, and shows a roughly linear increase of intensity with pH up to ~ 9 (Figure 4). Beyond this value, overlap of the free and complexed ^{11}B resonances prevent quantitation. The main pH-dependent effects include the titration of the two histidine residues, His12 and His119 in the active site [33] and titration of the boric acid. Specifically, for a total boric acid concentration B_T , the concentration of borate anion is given by:

$$B(OH)_4^- = \frac{B_T}{1 + 10^{pK_B - pH}} \quad [2]$$

where $pK_B = 9.0$ [34]. Since it is clear from the ^{11}B shift that the ternary 3'-deoxycytidine-2'-borate•RNase A complex involves the tetrahedral borate anion, the increasing $B(OH)_4^-$ concentration with increased pH can lead to an increase in the amount of ternary complex, even if the intrinsic binding affinity is reduced as the histidine residues deprotonate. In order to evaluate the change in binding affinity, we made up a sample at pH 7.0 with 46 mM boric acid – sufficient to produce the same level of $B(OH)_4^-$ that is present at pH 8.0 with 5 mM boric acid – and with all other constituents identical. For this sample, the concentration of ternary complex was determined to be 0.22 mM or 11 % of the RNase A. This compares with 3.5 % of the RNase A in the ternary complex for the sample containing 5 mM boric acid at pH 8.0 (Figure 4). This result thus supports the conclusion that the observed pH-dependent increase in ternary complex concentration results from the increased borate concentration in the sample, and further suggests that the intrinsic binding affinity of the active site for the borate ester is higher if the histidine residues are in the protonated form. This result is thus consistent with the higher affinity of RNase A for 2'-CMP and other phosphorylated nucleotides at lower pH [30,35,36].

Ligand Competition Study

As a further demonstration that the observed ^{11}B resonance corresponds to an active site complex that is structurally homologous with the complex formed with 2'-CMP, a ligand competition experiment was performed. 2'-CMP was titrated into a sample of the ternary complex with a composition similar to used for the study shown in Figure 1c. One constraint on this approach is that the pH must be high enough to allow significant ternary complex formation (Figure 4), but low enough to allow significant 2'-CMP binding. There is no reported K_i for 2'-CMP at pH 8.65, and at this pH value, we found little evidence for displacement of the ternary complex by a significant excess (10 mM) of 2'-CMP. The highest pH for which we found inhibition data is 7.5, at which 2'-CMP has been shown to inhibit RNase A with a $K_i = 0.7$ mM [30]. A series of ^{11}B NMR spectra obtained at pH 7.5 as a function of [2'-CMP] show the expected ligand competition effect (Figure 5a).

In order to analyze the ligand competition data, we first note that the study differs from the more typical example in the literature in which a second ligand is titrated into a sample with fixed concentrations of the enzyme and the first ligand (e.g., [37]). In the present case, analysis of the ligand competition experiment must in principle take into account the multiple equilibria that correspond to boric acid /OH⁻, borate/3'-deoxycytidine, borate/RNaseA, 3'-deoxycytidine/RNase A, 3'-deoxycytidine-2'-borate/RNase A, and 2'CMP/RNaseA, as well as several others. We begin by defining an apparent equilibrium constant for the binding of 3'-deoxycytidine-2'-borate (CMB) to the enzyme:

$$K_{E-CMB}^{app} = \frac{[CMB_f][RNase A_f]}{[CMB \bullet RNase A]} \quad [3]$$

The concentration of ternary complex can then be expressed as:

$$[CMB \bullet RNase A] = \frac{[CMB_f][RNase A_f]}{K_{E-CMB}^{app}} \approx \left(\frac{[CMB_f]}{K_{E-CMB}^{app}} \right) (E_o - [CMP \bullet RNase A]) \quad [4]$$

The first relation above is a rearranged version of Eq. [3]. For the second equality above, we assume that the concentration of CMB_f is maintained at constant value throughout the titration study as a result of the ability of 3-deoxycytidine and borate to buffer any changes that result from variable binding to RNase A. Such changes are in fact quite small since for all of the studies reported here, only a few percent of the RNase A exists as a ternary complex. Thus, the quantity $[CMB_f] / K_{E-CMB}^{app}$ may be considered to be constant throughout the 2'-CMP titration. The second approximate equality above also is based on the approximation that the effective concentration of free enzyme, RNase A_f, will be reduced by formation of the 2'CMP•RNase A complex. This approximation for RNase A_f neglects the variable fraction of enzyme bound to borate, 3'-deoxycytidine and to the 3'-deoxycytidine-2'-borate complex. The concentration of CMP•RNase A was determined from the relation:

$$[CMB \bullet RNase A] = \frac{1}{2} \left(E_o + CMP_o + K_c - \sqrt{[E_o + CMP_o + K_c]^2 - 4CMP_o E_o} \right) \quad [5]$$

using $K_C = 0.7$ mM [30], $E_o = 2$ mM, and the CMP_o concentrations indicated in Figure 5a. The resulting curve (Figure 5b) was fit by varying the adjustable (amplitude) parameter $[CMB_f] / K_{E-CMB}^{app}$. This study thus demonstrates that not only is 2'-CMP capable of displacing the complexed CMB from the active site of RNase A, but that the displacement is quantitatively consistent with the known inhibition constant of 2'-CMP at this pH. Thus, a significant variation of the K_C value used in the above equation resulted in a much poorer fit of the data. The conclusion that the CMB complex occupies the active site of RNase A is thus further supported. Furthermore, the observation that 2'-CMP is considerably more effective at displacing bound CMB at H 7.5 than at pH 8.65 suggests that the monoanionic borate ester has a relatively higher affinity for the unprotonated form of the enzyme than the dianionic CMP that predominates at high pH.

As is apparent from Figure 1b, no separate resonance corresponding to uncomplexed CMB is observed in the absence of enzyme. This results from a combination of low concentration and rapid exchange of the monodentate borate ester. This limitation precludes a determination of the apparent dissociation constant. We can make a very approximate estimate of the concentration of free CMB by assuming that the reaction rates with the 2'-hydroxyl group of

3'-deoxycytidine are identical to the reaction rates with water. Using this approach, we estimate the free CMB concentration at pH 7.5 as 1.5 μ M, giving $K_{E-CMB}^{app}(pH=7.5) \approx 82 \mu M$, or nearly a factor of 10 lower than the K_I for 2'-CMP at this pH. We emphasize, however, that since the resonance for uncomplexed CMB is not observed, this is a very rough number.

Structure of the RNase A•3'-deoxycytidine•borate complex

Crystallographic structures of the complex formed by RNase A with both cytidine-2'- and cytidine-3'-monophosphate have been determined [38,39]. The structure of the ternary RNase A•3'-deoxycytidine-2'-borate complex studied here is presumably homologous with the structure of the RNase A•2'-CMP complex. Based on this presumed homology, the important enzyme contacts are shown in Figure 6a. The position of the borate may be stabilized by hydrogen bond interactions with Gln11, His12, Lys41, and His119, although the smaller size of the borate would presumably lead to poorer binding, and some of the interactions apparent in the 2'-CMP complex could be absent. His12 is positioned by an N δ H hydrogen bond with Thr45 CO, so that the unprotonated N ϵ that predominates at higher pH is positioned to act as an H-bond acceptor with a borate OH. His119 is positioned by an N ϵ H that can hydrogen bond with Asp121, so that the unprotonated N δ also can accept an H-bond from a borate OH. Gln11 may present its carbonyl sidechain to the borate, also allowing it to act as an H-bond acceptor, however the orientation of the sidechain is probably maintained by an H-bond interaction with Lys41. The pK values for H12 and H119 are reported to be 6.0 and 6.5, respectively [33], so that they would be predominantly unprotonated under the conditions of most of the studies reported here. Thus, at pH 6.5 and above, the His12 and His119 residues probably function as H-bond acceptors, while Gln11 and Lys41 are H-bond donors to the borate. However, we emphasize that the protonation states of His12 and His119 in the ternary complex are unknown at present, but are shown in the unprotonated form in Figure 6a.

Superposition of the RNase A complexes formed with 2'-CMP (pdb code: 1ROB) and 3'-CMP (pdb code: 1RPF) show that the ligands are positioned differently, particularly due to a change in the glycosidic bond angle (Figure 6b). This change reduces the difference in position between the phosphate groups in the two structures; the two phosphorus atoms are separated by ~ 1 Å in the superimposed enzyme structures, but by ~ 3 -4 Å if the ribose rings are superimposed. The most significant structural variation in the RNase A is a change in position of the His119 sidechain in the 3'-CMP complex, which adopts an "A" rather than a "B" conformation [39].

The spectra in Figure 2 demonstrate the binding of the borate in a position optimal for ester formation with the 2'-hydroxyl group of 3'-deoxycytidine, but not with the 3'-hydroxyl group of 2'-deoxycytidine. This is consistent with the conclusion that the 3'-deoxycytidine-2'-borate complex mimics the stronger 2'-CMP inhibitor interactions [30,35,36]. This result is also consistent with the conclusion of Zegers et al. [39] that in the active enzyme His119 adopts the A conformation, which is observed in the complex with 2'-CMP, rather than with the B conformation, observed in the complex with 3'-CMP. Thus, these results demonstrate that formation of the borate ester is a sensitive probe of the phosphate ester binding site. In the ternary complex, ester formation appears to be selective for the active His119 conformation, and for the RNase A active site that is optimized to bind a 2'-phosphate or the homologous 2'-borate ligand.

Chemistry of borate ester Formation

Formation of borate esters has been studied in detail by Pizer and coworkers [34,40]. Ester linkages can be formed either as a result of addition chemistry to B(OH)₃, or by substitution chemistry involving reaction with B(OH)₃ (slower) or B(OH)₄⁻ (faster) [40]. If the incoming ligand is able to transfer a proton to one of the coordinated hydroxyl groups, allowing the

formation of water as a leaving group, the substitution reaction typically proceeds much more rapidly than the addition reaction. Based on these studies, the major pathway for the formation of the 2'-borate ester of 3'-deoxycytidine is illustrated in Scheme 1a.

The ternary RNase A•3'-deoxycytidine-2'-borate complex can in principle be formed by direct complexation of free 3'-deoxycytidine-2'-borate with RNase A, or serially by reaction of borate with the binary RNase A•3'-deoxycytidine complex or reaction of 3'-deoxycytidine with the binary RNase A•borate complex. Based on the inhibition constants for borate and 3'-deoxycytidine that are presented below, it is likely that the predominant pathway for ternary complex formation involves binding of the borate anion to the binary RNase A•3'-deoxycytidine complex, followed by the substitution reaction shown in Scheme 1. If this is the case, then it would be reasonable to expect that the borate anion would have a higher affinity for the more positively charged active site present at lower pH. This is also consistent with the qualitative conclusion summarized above that although the concentration of observable ternary complex increases with increasing pH, the increase results from the increased borate concentration and not from higher binding affinity. Thus, the affinity of the enzyme for the CMB appears to be greater at lower pH.

It is apparent that RNase A should be able to catalyze this reaction primarily by binding the 3'-deoxycytidine and borate reactants in close proximity. The Lys41 amino group shown in Figure 6 might further facilitate the substitution reaction by protonating a borate hydroxyl group and then accepting a proton from the 2'-hydroxyl on the 3'-deoxycytidine (Scheme 1b). Of course, the enzyme will be equally capable of catalyzing ester hydrolysis, and the concentration of free CMB should not be significantly influenced by the presence of the RNase A.

Formation of a ternary RNase A•cytidine•borate complex

As noted above, cytidine-2',3'-cyclic phosphate (cCMP) is used for assaying RNase A activity [31], and uridine-2',3'-cyclicvanadate ester inhibits RNase A [4,7-9], so that it appeared likely that cytidine-2',3'-borate (cCMB) would also bind to this enzyme. An experiment analogous to that shown in Figure 1 using cytidine instead of deoxycytidine is, however, not as readily interpreted, since the vicinal diols of cytidine support formation of stable 1:1 and 2:1 cytidine-borate complexes even in the absence of enzyme [41,42]. The ^{11}B NMR spectra of cytidine-borate mixtures obtained under conditions similar to those used for the studies with 3'-deoxycytidine were dominated by resonances arising from these complexes, so that it was necessary to explore different conditions to demonstrate RNase A binding of cCMB. Studies at lower pH and lower cytidine and borate concentrations, demonstrate a significant, ~3.5-fold increase in the intensity of the borate resonance ascribed to the cytidine-2',3'- $\text{B}(\text{OH})_2^-$ anion in the presence of RNase A, supporting the conclusion that this species is stabilized by binding to the enzyme (Figure 7). An apparent K_D for the interaction of $\text{B}(\text{OH})_3/\text{B}(\text{OH})_4^-$ with cytidine at pH 7 can be obtained from the concentrations and observed resonance intensities:

$$K_D^{\text{app}} = \frac{[\text{B}(\text{OH})_3 + \text{B}(\text{OH})_4^-][\text{Cytidine}]}{[\text{cCMB}]} = \frac{(0.98)(1.98)}{.02} = 97 \text{ mM} \quad [6]$$

This apparent dissociation constant obtained at pH 7 is relatively high, due in particular to the fact that most of the boric acid exists as $\text{B}(\text{OH})_3$. Using the above result, we can determine an apparent binding constant to the RNase A under these conditions:

$$K_{E-CB}^{app} = \frac{[cCMB][RNase\ A]}{[cCMB \bullet RNase\ A]} = \frac{(0.02)(1.95)}{0.05} = 0.78\text{ mM} \quad [7]$$

For the above calculation, it is assumed that the borate and cytidine concentrations are sufficient to maintain the free cytidine-2',3'-borate at .02 mM. This result compares with the reported Michaelis constant $K_m = 11\text{ mM}$ for cytidine-2',3'-cyclicphosphate at pH 7 [30]. Thus, the above calculations indicate that the cCMB binds to RNase A at pH 7 with more than tenfold the affinity of the substrate, cCMP. In contrast, the binding is much weaker than that reported for the uridine-vanadate complex, which has a $K_D \sim .5\text{ }\mu\text{M}$ [9]. The poor affinity of the active site of RNase A for cCMP at this pH appears to be related to the histidine pK values [33]. The K_m value for cCMP drops from 11 mM to 0.36 mM as the pH is reduced from 7 to 5 [30]. In addition, the NMR data were obtained at 5 °C, while the reported inhibition studies were at 25 °C.

Kinetic Analysis

The NMR data obtained for borate and 3'-deoxycytidine in the presence of RNase A predict that these two ligands should be competitive inhibitors of RNase A and exhibit positive cooperativity. RNase A activity was monitored by measuring the time-dependent disappearance of the ^{31}P resonance arising from cytidine-2',3'-cyclicphosphate (cCMP) and the parallel appearance of the cytidine-3'-phosphate resonance. Assays were performed at $T = 25\text{ }^\circ\text{C}$, 100 mM HEPES, pH 8.0, 10 mM cCMP, 1 μM RNase A. Initial studies using Tris as the buffer resulted in substantial synthesis of the 3'-Tris phosphate conjugate nucleotide, i.e. a product in which the Tris hydroxyl replaced water as the reacting nucleophile. Although this problem was not noted using HEPES buffer, determination of the pH values of HEPES and borate stock solutions before and after mixing indicates that even in this case, there is a significant borate-HEPES interaction. Under these conditions product formation, as judged by the appearance of the 3'CMP resonance, remained linear for at least 20 minutes, during which 0 – 15% of the cCMP was consumed. Due to the variable time required to obtain the first data point, a two parameter linear fit was used in which the curve was not forced to pass through 0. Competitive inhibition of cCMP hydrolysis by 3'-deoxycytidine or borate was analyzed using a non-linear fit of equation (1). These data fits yielded K_i values of 18 mM and 320 mM for 3'-deoxycytidine and borate, respectively. Calculation of these values assume the reported K_m of 41 mM for cCMP at pH 8, measured under similar conditions [30]. K_i for the inhibition of RNase A by borate is approximately 3 times greater than the inhibition constants for trypsin [26] and chymotrypsin [43] by borate. This weaker mimicry of the phosphate by boric acid reflects in particular the fact that at pH 8, only ~ 10 % of the acid exists as $\text{B}(\text{OH})_4^-$, which is presumably the closer homolog of phosphate, while for the serine proteases, the reaction is thought to involve attack of the active site serine on the boric acid species that mimics the trigonal carbonyl carbon. Since at pH 8, borate is ~ 10 % ionized, the K_i for $\text{B}(\text{OH})_4^-$ would be ~ 32 mM. A Yonetani-Theorell plot [44] of $1/v_i$ at a series of borate and 3'-deoxycytidine concentrations is shown in Figure 8. The set of curves yielded a cooperativity parameter of $\alpha = 0.1$, consistent with the positive cooperativity expected from the NMR data.

Discussion

Phosphorylation reactions are of critical importance to cellular function, playing roles in biochemical activation, cellular energetics, signal transduction, conformational switching, and receptor activation, among other processes. Since borate forms ester linkages non-enzymatically, it can theoretically mimic phosphate ester complexes. Further, it is reasonable

to expect that some phosphate ester-specific binding sites may stabilize structurally homologous, non-enzymatically formed borate esters.

In the present study, we sought to determine whether nucleoside-borate esters could mimic analogous phosphate esters as RNase A ligands by determining whether enzyme complexation could stabilize a ternary complex sufficiently to allow observation of the corresponding ^{11}B NMR resonance. As demonstrated here, formation of a ternary RNase A•3'-deoxycytidine-2'-borate complex is indicated by the observation of an ^{11}B resonance at -17.54 ppm (relative to external boric acid). Since this resonance is not observed for binary borate-RNase A or binary borate-3'-deoxycytidine mixtures, we conclude that the resonance arises due to RNase A stabilization of a 3'-deoxycytidine-2'-borate ester which structurally mimics the stable 2'-CMP inhibitor complex. One additional possibility that we considered was a structure in which the bound nucleoside "traps" an ionically complexed borate anion without actually forming an ester linkage. However, this possibility is effectively eliminated by the observation that 2'-deoxycytidine, which would be expected to exert a similar trapping effect, does not support formation of this complex, as judged by the absence of the upfield borate resonance (Figure 2). Further, stabilization of a complex with the borate esterified at the 2'-hydroxyl parallels the observation that 2'-CMP is a substantially stronger inhibitor than 3'-CMP [30,35,36]. The structural analogy between the RNase A complexes formed with 2'-CMP and with 3'-deoxycytidine-2'-borate is further supported by ligand competition studies performed at pH 7.5 (figure 5). The decrease in the observed intensity of the ^{11}B resonance assigned to the ternary complex follows the curve predicted by the $K_i = 0.7$ mM [30] reported for inhibition of Rnase A by 2'-CMP, consistent with competition for the active enzyme site.

In previous studies of borate-trypsin mixtures, we also have observed ^{11}B resonances for a binary borate-trypsin complex, which were demonstrated to arise from bidentate ester formation with two surface-exposed serine residues of the enzyme [45]. However, no analogous ^{11}B resonance was observed in the present study with ribonuclease A.

As summarized in the Introduction, phosphate mimicry by a range of oxyanions [1-13] and metal fluorides [14-19] has been extensively studied. In contrast, there has been little evidence in the literature suggesting borate ester formation as a phosphate-ester mimic. Borate has been reported to inhibit purine nucleotide pyrophosphotransferase, but no mechanism was proposed [46]. Reddi and Dreiling [47] observed that the activity of pancreatic ribonuclease was reduced by $\sim 50\%$ in the presence of 50 mM borate, and also that the effect of borate was reduced at higher concentrations of phosphate. These observations might result from borate mimicry of phosphate, but could also be interpreted on the basis of an ionic strength effect, since in general, ions reduce nucleic acid binding by competing for electrostatic binding interactions [48]. These observations are, however, consistent with the data presented here, and might result from a specific borate interaction(s) with phosphate binding site(s).

In contrast with the uridine-vanadate-RNase A and other vanadate-enzyme complexes in which the vanadate mimics a pentacoordinate phosphate transition state, the cytidine-borate complex mimics the complex formed with the cCMP substrate, while the 3'-deoxycytidine-2'-borate complex mimics the 2'-CMP inhibitor complex. The small size of the boron nucleus precludes the formation of pentacoordinate structures that would mimic putative associative transition state phosphate geometry. In this respect, borate phosphate mimicry contrasts with the analysis of borate complexes that form with serine proteases, in which the borate is believed to mimic the tetrahedral transition structure of the bound carbonyl group. Additionally, kinetic analyses indicate that borate binds to serine proteases in the unprotonated form [49], while as shown here, phosphate mimicry involves the borate anion.

Sugiyama et al. [23] recently have obtained chemical evidence for this type of phosphate ester mimicry by borate in the reaction catalyzed by L-rhamnulose-1-phosphate aldolase. In this example, reversible *in situ* formation of the borate ester of dihydroxyacetone, i.e. dihydroxyacetone borate is proposed to function as the donor substrate for the enzyme-catalyzed reaction with glyceraldehyde to form fructose. The authors point out that in addition, borate forms strong complexes with the fructose product, making the reaction functionally irreversible. Examination of the structure of a closely related enzyme, L-fuculose-1-phosphate aldolase, complexed with a phosphorylated inhibitor, phosphoglycolohydroxamic acid, shows that the phosphate binding site involves apparent H-bond formation with serine, threonine and asparagine residues, and with backbone NH groups [50]. This set of active site interactions is thus rather different from those involved in RNase A – borate complexation, suggesting that phosphate mimicry is not limited to sites with a specific group of receptor binding ligands.

In addition to potential synthetic applications, the ability of proteins to stabilize borate esters could also result in stabilization of serine-borate or tyrosine-borate esters by receptors for phosphorylated peptides. Examination of reported structures suggests that many of these complexes involve salt bridges between phosphate and arginine, while this residue is not involved in active site borate binding in RNase A. In this context, we note that the reported crystal structure of allophycocyanin contains borate anions that are positioned to interact with Ser18 and Arg24 residues, supporting the feasibility of phosphate mimicry by borate at this type of binding site [51]. More generally, formation of borate esters can provide insight into structural and activation aspects of homologous phosphate-esters. In the present case, the relative stability of the ternary complex formed from 3'-deoxycytidine contrasts with the stability of the complex formed from 2'-deoxycytidine, predicting that 2'-CMP will be a better RNase A ligand than 3'-CMP, consistent with the available literature. This type of approach could presumably be used to explore the binding of borate esters to other proteins, leading to predictions of phosphorylated ligand affinity.

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List of abbreviations

2'-CMP	cytidine-2'-monophosphate
3'-CMP	cytidine-3'-monophosphate
cCMB	cytidine-2',3'-cyclic borate
cCMP	cytidine-2',3'-cyclic phosphate
3'-dC	3'-deoxycytidine
CMB	3'-deoxycytidine-2'-monoborate ester
γ GT	γ -glutamyl transpeptidase
NMR	nuclear magnetic resonance
RNase A	ribonuclease A

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Figure 1.

^{11}B NMR spectra for RNase A - borate - 3'-deoxycytidine mixtures. a) 5 mM borate, 2 mM RNase A; b) 5 mM borate, 10 mM 3'-deoxycytidine; c) 5 mM borate, 10 mM 3'-deoxycytidine, 2 mM RNase A. All samples contained 50 mM HEPES, pH 8.65, and the spectra were obtained at $T = 5^\circ\text{C}$. Total acquisition time for each spectrum was 12.5 h, corresponding to 30,000 transients.

Figure 2.
 ^{11}B NMR spectra. a) 5 mM borate, 2 mM RNase A, 10 mM 2'-deoxycytidine; b) 5 mM borate, 2 mM RNase A, 10 mM 3'-deoxycytidine. All samples contained 50 mM HEPES, pH 8.65, and the spectra were obtained at $T = 5^\circ\text{C}$.

Figure 3.

Effect of temperature on the stability of the ternary complex. ^{11}B spectra of samples containing 5 mM borate, 2 mM RNase A, 60 mM 3'-deoxycytidine at a) $T = 5$, b) $T = 15$, c) $T = 25$ °C. Samples also contained 50 mM HEPES, pH 8.65.

Figure 4.

Percent of RNase A forming the RNase A•3'-deoxycytidine•borate ternary complex, determined as a function of pH. The sample contained 2 mM RNase A, 5 mM borate, 10 mM 3'-deoxycytidine, in 50 mM HEPES. The fractional intensity of the ^{11}B signal arising from the ternary complex was calculated, and the results converted to % of RNase A based on the total borate and RNase A concentrations in the sample.

Figure 5.

Ligand competition study. a) A series of ^{11}B spectra for a sample initially containing 2.0 mM RNase A, 5 mM borate, 10 mM 3'-deoxycytidine in 50 mM HEPES, pH 7.5. Spectra a through e were obtained with [2'-CMP] concentrations of 0, 1, 2, 5, and 10 mM, respectively. Sample temperature was 5°C . Only the region containing the resonance of the ternary complex is shown. b) Fit of the ligand competition data. The fraction of RNase A in the ternary complex was computed from the fraction of bound borate determined from the ^{11}B resonances.

Figure 6.

Active site interactions. a) Interactions of active site RNase A residues with 3'-deoxycytidine-2'-borate are modeled on the basis of the crystal structure of the RNase A complex with cytidine-2'-phosphate (pdb code 1ROB, Lisgarten et al. [26]). b) Overlay of the active site structures for RNase A complexes with 2'-CMP (1ROB) and with 3'-CMP ([27], 1RPF). The 1ROB structure is shown using CPK colors, while for the 3'-CMP complex, the protein residues are green and the 3'-CMP is cyan. Some of the H-bond interactions in the 1ROB structure are indicated as red dotted lines.

Figure 7.

^{11}B spectra of the RNase A complex with cytidine-2',3'- B(OH)_2^- . ^{11}B NMR spectra of samples containing 1 mM borate, 2 mM cytidine in 50 mM HEPES, pH 7.0 were obtained in the presence (a) or absence (b) of 2 mM RNase A. Other conditions were: 30,000 transients, corresponding to a total acquisition time of 12.5 h, $T = 5^\circ\text{C}$. The resonance at $\delta = -12.3$ ppm attributed to cytidine-2',3'-borate exhibits a very different shift than the resonance arising from the borate monoesters (e.g., Figure 1). The resonance of the 2:1 cytidine-borate complex, which is barely observable under the conditions of the study, is at -7.6 ppm.

Figure 8.

Kinetic analysis of RNase A – borate – 3'-deoxycytidine mixtures. Sample contained 1 μ M RNase A, 10 mM cCMP, 100 mM HEPES, pH 8.0, as well as 3'-deoxycytidine and borate at the concentrations indicated. Initial velocity was determined from the intensity of the ^{31}P resonance of the 3'-CMP product, 3'-dc (3'-deoxycytidine).

Scheme 1.

a) Substitution reaction of 3'-deoxycytidine with borate. b) Possible Lys41 facilitation of borate substitution chemistry on RNase A.