Boron-11 Pure Quadrupole Resonance Investigation of Peptide Boronic Acid Inhibitors Bound to α-Lytic Protease†

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ABSTRACT: Pure quadrupole resonance is a potentially useful spectroscopic approach to study the coordination of quadrupolar nuclei in biological systems. We used a field-cycling NMR method to observe boron pure quadrupole resonance of two peptide boronic acid inhibitors bound to α-lytic protease. The method is similar to our earlier field-cycling experiment [Ivanov, D., and Redfield, A. R. (1998) *Z. Naturforsch. A 53*, 269−272] but uses a simple Hartmann−Hahn transfer from proton to ^{11}B before field cycle and direct ^{11}B observe after it. Pure quadrupole resonance is sensitive to the boron coordination geometry. For example, trigonal boron in neutral phenylboronic acid, which was used as a model compound, resonates at 1450 kHz, while the resonance of the tetrahedral phenylboronic acid anion appears at approximately 600 kHz. In the complex of the MeOSuc-Ala-Ala-Pro-boroVal inhibitor with the enzyme the quadrupole resonance signal was observed at 600−650 kHz, which indicates tetrahedral boron coordination in the active site. The quadrupole frequency of the MeOSuc-Ala-Ala-Pro-boroPhe enzyme−inhibitor complex, in which a boron−histidine bond is known to be formed, was found to be the same within experimental error as in the MeOSuc-Ala-Ala-Pro-boroVal enzyme−inhibitor adduct, suggesting that the boron coordination geometry in the enzyme−MeOSuc-Ala-Ala-Pro-boroPhe adduct is also close to tetrahedral.

Peptide boronic acids form a class of potent and specific competitive inhibitors of serine proteases. Early investigations indicated that boronic acid inhibitors form reversible covalent adducts with the active site serine of the enzyme, in which the tetrahedral geometry of boron coordination mimics the high-energy tetrahedral geometry on the reaction center carbon during the hydrolysis reaction (*1*, *2*). For this reason boronic acid inhibitors are often considered to be transition state analogue inhibitors. Peptide boronic acid inhibitors are usually tri- or tetrapeptides with a boronic acid group in place of the C-terminal carboxylate (*3*). When the peptide composition is based on the substrate specificity of a particular protease, the inhibitor combines the favorable geometry of the adduct formed by the boron atom in the active site with the enzyme−substrate interactions of the peptide chain. For example, α-lytic protease is a bacterial protease which normally cleaves after small hydrophobic amino acids, and MeOSuc-Ala-Ala-Pro-boroVal[1] was found to be a potent inhibitor of the enzyme with a K_i of 6.4 nM (*4*). On the other hand, when the peptide composition does not satisfy the specificity requirements of the protease, the geometry of the boron coordination in the active site can be perturbed. ^{15}N NMR investigations of the MeOSuc-Ala-Ala-Pro-boroPhe peptide bound to α-lytic protease revealed that it forms a boron−nitrogen bond with the active site histidine (*5*). The subsequent X-ray studies of several peptide boronic acid inhibitors bound to α-lytic protease confirmed that the binding modes of the boroVal and boroPhe inhibitors were different (*6*). The MeOSuc-Ala-Ala-Pro-boroVal inhibitor formed the expected tetrahedral adduct with the active site serine, while the MeOSuc-Ala-Ala-Pro-boroPhe was bound in an unusual bridging conformation, in which, in addition to a boron−serine bond, a boron−histidine bond was present. The geometry of boron coordination appeared closer to trigonal pyramidal than to tetrahedral, with the active site histidine forming the axial bond to boron. This unusual coordination geometry was contested in a later ^{11}B solution NMR study, in which the ^{11}B chemical shifts of the MeOSuc-Ala-Ala-Pro-boroVal and the MeOSuc-Ala-Ala-Pro-boroPhe inhibitors bound to α-lytic protease were found to be essentially the same (*7*).

The mechanism by which the side chain of a boronic acid compound affects the geometry adopted by the boron in the

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[1] Abbreviations: PQR, pure quadrupole resonance; PEG, poly(ethylene glycol); THF, tetrahydrofuran; RF, radio frequency; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MeOSuc, methoxysuccinyl. BoroVal and boroPhe indicate that the carboxylate of the C-terminal amino acid residue is replaced with $B(OH)_2$.

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active site is of interest because of the potential of boronic acids as drugs. To provide additional spectroscopic information about the boron coordination, we applied a modification of a solid-state field-cycling NMR method (*8*) to detect boron pure quadrupole transitions of the MeOSuc-Ala-Ala-Pro-boroVal and the MeOSuc-Ala-Ala-Pro-boroPhe inhibitors bound to α-lytic protease. Pure quadrupole resonance (PQR), also known as nuclear quadrupole resonance (NQR), measures the electric quadrupole interaction of the nuclear electric quadrupole moment with the gradient of the electric field produced by the surrounding charges. The sensitivity of the quadrupole resonance to the changes in the electronic environment of the boron provides an alternative spectroscopic method to monitor boron coordination.

EXPERIMENTAL PROCEDURES

α-Lytic protease was purified from *Lysobacter enzymogenes* cultures using ion-exchange chromatography (*9*). The protein was concentrated using stirred cell concentrators and a 10 kDa cutoff filtration membrane. Protein concentration was determined from an activity assay monitoring hydrolysis of Suc-Ala-Ala-Pro-Ala-pNA substrate.

The synthesis of the MeOSuc-Ala-Ala-Pro-L-boroVal and MeOSuc-Ala-Ala-Pro-D,L-boroPhe inhibitors was as previously described (*3*); the latter was kindly provided by Dr. C. A. Kettner. The two protein−inhibitor samples were prepared as lyophilized powders. First, 6 mg of the inhibitor was dissolved in 0.5 mL of methanol, and then the solution was injected into 140 mL of 50 mM, pH 7.5, Tris-HCl buffer and incubated for 1 h to remove the protecting pinacol group (*4*). After that, about 10 mL of a 1 mM solution of α-lytic protease in 50 mM, pH 7.5, Tris-HCl buffer was added to the inhibitor solution to give a roughly 1 to 1 protein−inhibitor ratio. The protein was incubated with the inhibitor for another 25 min and then concentrated to ∼5 mL in a stirred cell concentrator. Sixty milligrams of 3250 MW PEG was added to the concentrated sample as a cryoprotectant, and the sample was shell frozen using liquid nitrogen and lyophilized for 24 h. The lyophilized powder was loaded into a sample holder and stored in liquid nitrogen between experimental runs.

Samples of phenylboronic acid were used as model systems. A partially titrated sample of phenylboronic acid was prepared as a frozen solution containing 40% glycerol, 100 mM phenylboronic acid, and 50 mM NaOH. A complex of phenylboronic acid with imidazole was prepared as a solution of the two compounds in an aprotic solvent (*5*). Phenylboronic acid (100 mM) and imidazole (100 mM) were dissolved in deoxygenated THF and frozen using liquid nitrogen. We could not use chloroform as a solvent, because chlorine, which has a large nuclear quadrupole moment, interferes with the field-cycling experiment by producing short relaxation times.

The boron pure quadrupole spectra of the lyophilized protein samples were collected using a variant of a field-cycling NMR technique (*10, 11*). The use of the proton NMR signal as the readout signal after the field cycle, as demonstrated previously, did not work in frozen protein solutions or lyophilized protein powders because of, among other reasons, fast proton relaxation at low fields not seen in our earlier nonprotein experiments (*8*). Instead, we developed a modification of the method which uses Hartmann−Hahn transfer between the proton and ^{11}B before the field cycle and ^{11}B central transition detection after the cycle (*12*). The method uses the central transition signal at high field to indirectly detect the low-field pure quadrupole transitions. A simplified diagram of the experiment is shown in Figure 1. The field cycle was performed by pneumatically shuttling the sample inside a variable temperature flow dewar (*8*). The operating temperature was between 10 and 30 K along the sample transfer path. The relaxation delay between the field cycles was 10 s. The boron readout signal was observed at high field (11.4 T) after a 10 μs 90° pulse. The field cycle was repeated many times, incrementing the frequency of the search RF pulse from cycle to cycle to eventually cover the range of the expected quadrupole frequencies. The spectra in Figure 3 are the Fourier-transformed boron readout signal intensity plotted as a function of the low-field search RF frequency. Pure quadrupole transitions appear as decreases in the readout signal intensity. The low field was set to 50 G, and the parameters of the search RF irradiation at zero field can be found in the figure captions. The low field of 50 G hardly broadens the ^{11}B quadrupole resonance and was found to give a better signal than at zero field, for reasons that are not fully understood. A complete description of the method will appear. The spectra of the protein-bound inhibitors in Figure 3 were collected in approximately 24 h. Each data point in the spectrum is a sum of approximately 300 field cycles.

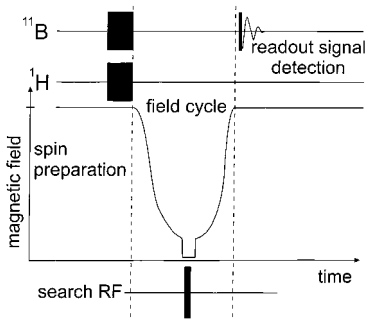

FIGURE 1: Timing diagram of a field-cycling NMR experiment. The strong readout signal of the boron central transition at high field is used to indirectly detect the low-field pure quadrupole transitions produced by the RF pulse at low field. The field cycle is repeated many times, incrementing the frequency of the search RF pulse from cycle to cycle. When the peak of the Fourier-transformed readout signal is plotted as a function of the low-field search RF frequency, pure quadrupole transitions appear as decreases of the readout signal intensity. The central transition ^{11}B readout pulse was a 10 μs 90° pulse. The initial cross-polarization intensities were the same for ^{11}B, as well as for protons (in frequency units), and were both 30 kHz off-resonance. Because of cross-relaxation via level crossing below about 1000 G, the field has to be turned off and on rapidly, in about 1 ms, below this field.

RESULTS

Phenylboronic acid acts as a Lewis acid, forming a covalent adduct with the hydroxide ion (Figure 2). The boron coordination geometry changes from planar trigonal in the neutral phenylboronic acid to tetrahedral in the four-coordinate phenylboronic acid anion. Figure 3A shows the spectrum of phenylboronic acid 50% titrated with NaOH. The higher frequency peak centered at 1450 kHz in the spectrum corresponds to the trigonal boron coordination,

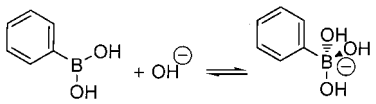

FIGURE 2: Lewis acid−base equilibrium of the phenylboronic acid ionization. Boron coordination is planar trigonal in neutral phenylboronic acid and is tetrahedral in the anion.

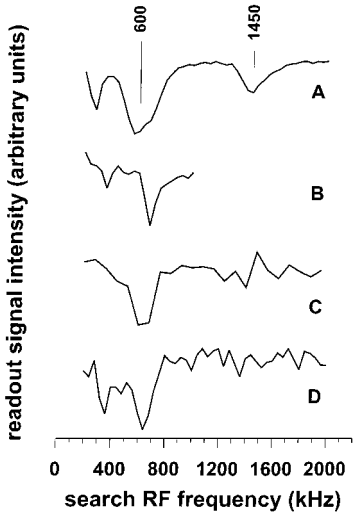

FIGURE 3: Boron pure quadrupole spectra. (A) Frozen solution of a roughly equimolar mixture of neutral phenylboronic acid and phenylboronic acid anion. The low-field search RF pulse amplitude was 20 G, and the pulse was 200 μs long. (B) Phenylboronic acid−imidazole adduct in tetrahydrofuran. The search RF pulse was 100 μs at about 16 G. (C) Pure quadrupole spectrum of α-lytic protease with a MeOSuc-Ala-Ala-Pro-boroVal inhibitor bound. The search RF pulse was 200 μs at 10 G. (D) Spectrum of MeOSuc-Ala-Ala-Pro-D,L-boroPhe inhibitor bound to α-lytic protease. The search RF pulse was 100 μs long at 16 G.

while the peak at 600 kHz is the resonance of the four-coordinate anion. The 1450 kHz peak is the only peak present in the spectrum at low pH, while the 600 kHz peak appears and becomes more intense as the pH is raised by titration with NaOH. The weaker peak at 280 kHz is probably the result of simultaneous boron−proton spin flips and is seen at high RF power. The weak transitions at ~360 kHz in spectra B and D of Figure 3 have the same origin. The frequencies of these transitions depend on the search RF power. They are presumably analogous to those of the "solid effect", which is commonly used to polarize nuclei in rigid solids by RF power, applied off-resonance, to electron spins, to produce highly polarized targets for nuclear physics experiments. In the present case the protons presumably play the same role as electrons in the solid effect.

To obtain ^{11}B quadrupole coupling information in a model compound, in which a boron−nitrogen bond is present, we collected field-cycling spectra of a frozen equimolar solution of phenylboronic acid and imidazole in tetrahydrofuran. A boron−nitrogen bond similar to the one found in the complex of the MeOSuc-Ala-Ala-Pro-D,L-boroPhe inhibitor with α-lytic protease is reportedly formed in an equimolar mixture of phenylboronic acid and imidazole in aprotic solvent, based on the ^{15}N solution NMR data (*5*). The boron pure quadrupole transition of the phenylboronic acid−imidazole complex (Figure 3B) appears at 680 kHz and is the same, within the line width, as that of the quadrupole transition of the four-coordinate phenylboronic acid anion in Figure 3A.

The spectrum of the MeOSuc-Ala-Ala-Pro-L-boroVal bound inhibitor (Figure 3C) has a single peak at ~650 kHz, clearly indicating tetrahedral boron coordination in the active site, as expected. The spectrum was collected in approximately 24 h and is the result of about 1000 field cycles for each frequency point of the search RF.

The spectrum of the MeOSuc-Ala-Ala-Pro-D,L-boroPhe bound to the enzyme is shown in Figure 3D. There are two different stereoisomers of the inhibitor present in the sample, but there is just one boron pure quadrupole peak observed in the spectrum at 640 kHz. The extra peak at 360 kHz is not the result of the presence of two inhibitor stereoisomers, but as in the phenylboronic acid spectra it is the artifact due to simultaneous boron−proton transitions occurring at higher search RF power (above). Although the L and the D stereo isoforms of peptide boronic acid inhibitors are known to bind the enzyme with different affinity (*4*), both isoforms are expected to be bound to the enzyme at the high concentrations used to prepare the samples. The broad line probably masks the difference in the quadrupole transition frequency of the two bound isoforms.

DISCUSSION

Pure quadrupole resonance is an attractive tool for the study of active site species in proteins because it is sensitive to both covalency and ionic surroundings. There has been only one report of PQR in a protein, for copper in reduced Cu−Zn superoxide dismutase (*13*). A recent landmark experiment determined the quadrupole interaction in a purely high-field experiment for ^{67}Zn ligated to a small protein (*14*) from a line-shape analysis of the central NMR transition (*15*). Our indirect detection field-cycling strategy (Figure 1) allows high-sensitivity detection of the quadrupole transitions even below 1 MHz. As far as we know, our results are the first observations of such low-frequency quadrupole transitions in a protein.

Field-cycling pure quadrupole resonance is a useful alternative to the high-field NMR of the central transition (*15*) for determination of quadrupole parameters. For a small quadrupole interaction, chemical shift anisotropy and dipole−dipole coupling can mask the quadrupolar singularities of the central transition. Overlapping high-field NMR lines would also complicate interpretation of the quadrupolar line shapes if several different binding sites or species were present. In a pure quadrupole spectrum species with sufficiently different quadrupole parameters appear at very different frequencies. Figure 3A, for example, shows two distinct peaks corresponding to the trigonal geometry of boron coordination in neutral phenylboronic acid and to the tetrahedral coordination geometry in the phenylboronic acid anion in a sample containing both species. Boron quadrupolar parameters have been determined for a number of molecules (*16*), but the previous data are limited to inorganic compounds or to organic compounds dissimilar to the boronic acids.

Spin $^3/_2$ nuclei have only one pure quadrupole transition, the frequency of which is a function of the quadrupole coupling constant and the asymmetry parameter. Unfortunately, no reasonable estimate of the asymmetry parameter can be made from the observed transition frequency unless specialized methods are used (*17*). These methods might, in

principle, be combined with field-cycling quadrupole resonance. Although the transition frequency cannot be easily interpreted in terms of the quadrupole coupling constant and the asymmetry parameter, the quadrupole transition can be used as a spectroscopic signature of a particular structure, analogous to the common use of chemical shift, as is seen in Figure 3A. In addition, the asymmetry parameter can be assumed small or zero when the coordination geometry is expected to have approximately axial symmetry, as in the case of the four-coordinate boron in the phenylboronic acid anion.

The spectra of the phenylboronic acid adducts (Figure 3A,B) show, on one hand, that the boron pure quadrupole resonance can be used to distinguish between the trigonal-planar boron coordination of the free phenylboronic acid and the four-coordinate boron of the adducts. On the other hand, the difference between the hydroxide ion adduct and the imidazole adduct of phenylboronic acid is less than the resolution of the method. Although the precision with which the quadrupole parameters can be determined may be higher for this technique than for the high-field methods, broad lines still limit the resolution of the approach. The line width is presumably due to static heterogeneity of the size of the quadrupole interaction resulting from minor structural heterogeneity of the frozen protein, as well as magnetic dipolar broadening (*12*).

The quadrupole resonance at ~650 kHz in the spectrum of the MeOSuc-Ala-Ala-Pro-L-boroVal inhibitor bound to α-lytic protease clearly indicates tetrahedral boron coordination in the active site of the enzyme. The quadrupole resonance of the bound MeOSuc-Ala-Ala-Pro-D,L-boroPhe inhibitor appears at essentially the same frequency as the resonance of the bound boroVal inhibitor. The observed difference, if any, is much smaller than the difference between the trigonal and the tetrahedral boron coordination. The boron transition frequencies of the two bound inhibitors appear identical to the transition frequencies of the four-coordinate boron in the adducts of phenylboronic acid. The most likely interpretation of these results is that the boron coordination geometry in the active site of the enzyme is close to tetrahedral for both the boroPhe and the boroVal inhibitors. A similar interpretation was proposed in a ^{11}B solution NMR study, in which no ^{11}B chemical shift difference was observed between the two bound inhibitors (*7*). The apparent contradiction of the spectroscopic results with the X-ray structures of the enzyme−inhibitor complexes might ultimately be resolved by computational approaches. The ab initio calculations of the electric field gradients are believed to be more reliable than the computational predictions of the chemical shift values. It would be interesting to see if the predicted difference between the electric field gradient from the ab initio calculations, for the tetrahedral and the pyramidal-trigonal coordination seen in the X-ray structure, is big enough so that they should have been resolved in the present study. It is also possible that the trigonal adduct observed in the X-ray study is a minor species in solution which, however, yielded better crystals.

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