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Probing the general time scale question of boronic acid binding with sugars in aqueous solution at physiological pH

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ABSTRACT

The boronic acid group is widely used in chemosensor design due to its ability to reversibly bind diol-containing compounds. The thermodynamic properties of the boronic acid–diol binding process have been investigated extensively. However, there are few studies of the kinetic properties of such binding processes. In this report, stopped-flow method was used for the first time to study the kinetic properties of the binding between three model arylboronic acids, 4-, 5-, and 8-isoquinolinylboronic acids, and various sugars. With all the boronic acid–diol pairs examined, reactions were complete within seconds. The k_{on} values with various sugars follow the order of D-fructose > D-tagatose > D-mannose > D-glucose. This trend tracks the thermodynamic binding affinities for these sugars and demonstrates that the 'on' rate is the key factor determining the binding constant.

1. Introduction

The boronic acid functional group has been widely used in the design and synthesis of sensors for carbohydrates and other diol-containing compounds.[1–7] Such applications rely on the known thermodynamic property of boronic acid to form a tight complex with a diol moiety. Such thermodynamic properties have been extensively studied in terms of the magnitude of the binding constants with various sugars and factors that affect binding.[8,9] Equally important in this field is the understanding of the kinetic issues in the boronic acid–diol binding process. For example, boronic acid has been used in the design and synthesis for the real time determination of glucose concentration.[10] However, if binding takes minutes to reach equilibrium, then real time may not necessarily mean instantaneous. The same question applies to essentially all chemosensor design, that is, the issue of response time. It is generally believed that boronic acid–diol binding rapidly reaches equilibrium. By using the fluorescence method, we normally wait for no more than 10 min after mixing these two components before taking measurements, indicating that equilibrium is reached within the time scale of minutes. However, there are reports that it takes hours before equilibrium is reached.[11,12] There have been some kinetic studies of the binding between boric acid/boronic acids and diol by using NMR,[13–15] stopped-flow[16,17] and temperature jump[15,18–20] techniques. However, there has never been a detailed study of exactly how fast the binding process occurs between boronic acids with carbohydrates. Such information is critical to sensor design, again, because response time is very important to chemosensor applications. For this reason, we were interested in studying the kinetics of the boronic acid–diol binding process. Our past experiences indicated that such reactions were complete within minutes. For such fast reactions, we chose to use stopped-flow experiments to capture signal changes on the millisecond scale. In designing these experiments, we needed boronic acids that can show intrinsic signal changes upon binding. We have recently described a series of isoquinolinylboronic acids, which show large fluorescent signal changes upon binding.[4] The boronic acids chosen for this study include 4-, 5-, and 8-isoquinolinylboronic acids (4-IQBA, 5-IQBA and 8-IQBA). Their abilities to change fluorescence upon binding allowed for the direct detection of the binding event by using a fluorometer coupled with a stopped-flow instrument. As for diols, we selected a number of commonly seen sugars such as D-glucose, D-fructose, D-mannose, and D-tagatose. Below, we describe our studies in detail.

2. Materials and methods

All reagents were purchased from Aldrich and Frontier Scientific. For thermodynamic binding experiments, an RF-1501 Shimadzu fluorometer was used. For stopped-flow experiments, an Applied Photophysics RX2000 Rapid Mixing stopped-flow unit with FluoromaxIIII fluorometer (Horiba) was used. The dead time for this instrument is 0.05 s. All kinetic experiments were conducted in phosphate buffer (0.1 M) at pH 7.4 and at room temperature. Kinetic measurements were performed under pseudo first-order conditions. In a fixed concentration of IQBAs, different

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concentrations of sugars were mixed within a short time period. All the reaction curves were fitted using formula (1) in Origin 8. Using formula (2), K_{obs} can be calculated. Values for k_{on} and k_{off} were calculated using formula (3) by varying [S], the substrate concentration.

$$y = y_0 + Ae^{x/t1} \quad (1)$$

$$K_{obs} = -1/t1 \quad (2)$$

$$K_{obs} = k_{off} + k_{on}[S] \quad (3)$$

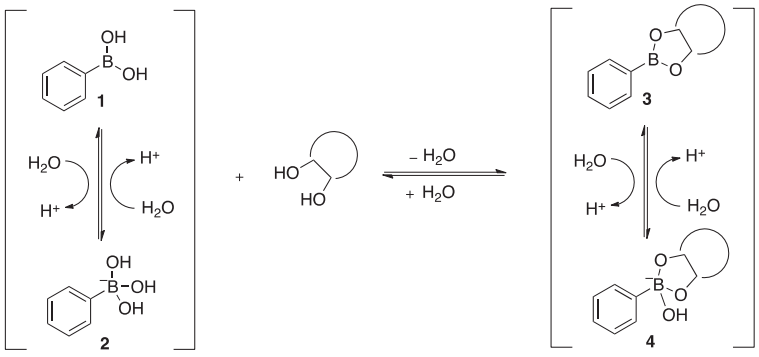
3. Results and discussion

In studying the kinetic properties of the binding processes, it is important to have a general understanding of the structural properties of boronic acid and the steps involved in boronic acid binding to a diol. It is well known that boronic acids (**1**, Scheme 1) are weak Lewis acids due to the open shell on the boron atom. It can react with a protic solvent molecule such as water to yield the tetrahedral boronate (**2**, Scheme 1) while releasing a proton. The presence of this open shell is also responsible for the ability of boronic acid to bind reversibly with diols,[8,21] single hydroxyl groups,[22] α-amino acids,[23] α-hydroxy acids[24] and other Lewis bases.[25] It is also important to note that boronic ester (**3**) is also an acid and can undergo the same reaction with water as boronic acid. Such a reaction leads to the formation of the tetrahedral boronate ester (**4**).

The goal for this study is very straightforward, that is, the understanding of the reaction kinetics in the formation of a boronic acid–diol complex. Therefore, we set out to determine the 'on and off' rates for each IQBA and sugar pair as described in Section 1. The results are shown in Table 1. However, before any discussion of the implications of the results obtained, it is important to look at the possible steps involved in the binding process (Scheme 2). As discussed earlier, boronic acid can exist in two states: neutral trigonal (**1,** Scheme 1) and tetrahedral anionic (**2**) forms. Depending on the pK_a of the boronic acid in question and solution pH, the ratio of these two forms changes. Binding of either the trigonal or the tetrahedral form with a diol can lead to the formation of two ester forms as well: the trigonal neutral (**3**) and the tetrahedral anionic (**4**) forms. Each reaction may involve many steps. In a simplistic approach, it would involve only two steps. If one examines this in detail, one can readily see that there may be multiple steps involving more than one pathway. Thus, the kinetic pictures can be quite complex. This is coupled with the fact that the ionization state of the isoquinolinyl nitrogen may change as well during the binding process, adding even more complications to this already complex process. It is important to note that the stopped-flow method may not be fast enough to allow for the examination of each individual step. However, for the purpose of studying the general time scale of the reaction, the method used is sufficient.

In studying the reaction kinetics, significant fluorescent intensity changes were observed, as expected, upon mixing of the various sugars with the three model boronic acids. Figure 2 shows a few representative curves for the binding between 8-IQBA and various sugars. One can quickly see that essentially all reactions reach equilibria within seconds. For example, for the reaction between 8-IQBA and D-fructose, the reaction reached equilibrium within about 4 s. On the other hand, it took about 14 s for the reaction between D-glucose and 8-IQBA to reach equilibrium. Such results already suggest that the difference in binding constants among different sugars might be attributed to variations in the 'on' rates, because D-fructose is known to bind more tightly with arylboronic acids than D-glucose.

To gain a quantitative sense of the kinetics, the k_{on} values for all binding pairs were calculated. Experiments were carried out in the concentration range from K_d to 10-fold K_d for each sugar-boronic acid pair. The k_{on} values ranged from 0.2 M^{-1} s^{-1} for the 4-IQBA-glucose pair to 287 M^{-1} s^{-1} for the 8-IQBA-fructose pair. This represents about 1500-fold differences among the sugars and boronic acids used. Earlier, there have been studies of the kinetic process of inter-conversion between the trigonal boronic acid and the tetrahedral boronate using a low-temperature stopped-flow

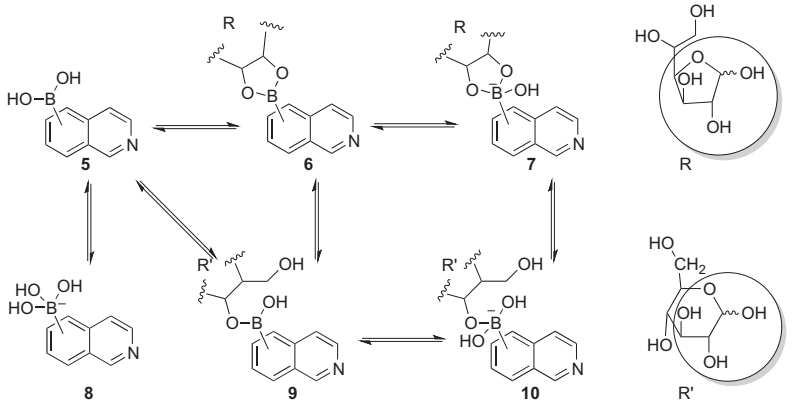


Scheme 1. Binding equilibrium of phenylboronic acid with a diol.

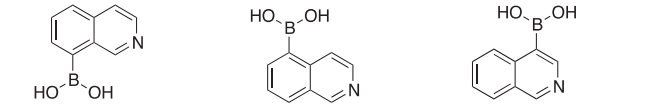
Table 1
k_{on}, k_{off} and apparent association constants (K_a) of isoquinolinylboronic acids with representative sugars[4]

D-Sugar	8-IQBA				5-IQBA				4-IQBA			
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	Calculated K_a (M^{-1})	Literature K_a[a] (M^{-1})	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	Calculated K_a (M^{-1})	Literature K_a[a] (M^{-1})	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	Calculated K_a (M^{-1})	Literature K_a[a] (M^{-1})
Fructose[a]	287	0.38	755	1493 ± 25	44	0.21	209	1432 ± 242	59	0.18	328	2170 ± 184
Tagatose[b]	169	0.36	469	1183 ± 367	34	0.22	155	1193 ± 121	35	0.21	167	1651 ± 11
Mannose[b]	17	0.38	45	84 ± 16	5	0.35	14	10 ± 6	4	0.18	22	85 ± 41
Glucose[a]	0.6	0.13	5	46 ± 12	0.46	0.08	6	42 ± 6	0.2	0.02	10	25 ± 7

[a] K_a was determined using a fluorescence method. Binding studies were conducted in phosphate buffer (0.1 M) at pH 7.4 (all experiments were duplicated).
[b] Binding constants from published literature.[4]



Scheme 2. Possible processes for the binding between isoquinolinylboronic acid and sugar.



8-isoquinolinylboronic acid (8-IQBA) 5-isoquinolinylboronic acid (5-IQBA) 4-isoquinolinylboronic acid (4-IQBA)

Figure 1. Structures of isoquinolinylboronic acids.

spectrophotometer. The rates were determined to be 7.9×10^4 M^{-1} s^{-1} at 25 °C,[17] indicating that the inter-conversion between trigonal and tetrahedral form is much faster than the actual binding process. Of course, such results make sense. In addition, there have also been studies of borate complexation processes using temperature jump method.[18–20] The rate is 10.7 M^{-1} s^{-1} for the reaction between boric acid and H_2O at 25 °C. To put the observed k_{on} values in perspective, a second order rate constant of 287 M^{-1} s^{-1} gives a half life of 3.5 s at 1 mM of starting material concentration, and 3484 s at 1 μM starting material concentration. In contrast, a second order rate constant of 0.2 M^{-1} s^{-1} gives a half life of 5000 s at 1 mM of starting material concentration and 5 s at 1 M starting material concentration. On the other hand, diffusion controlled reactions have an upper limit of 10^{10} M^{-1} s^{-1}.[26] Such numbers serve as very useful guidelines in estimating the time it takes for similar reactions to reach equilibrium, and thus the response time in chemosensor applications. As expected, the binding rate was structure-sensitive for each boronic acid. For example, D-fructose is about 500-fold faster in k_{on} than D-glucose when binding with 8-IQBA, 100-fold faster when binding with 5-IQBA, and 300-fold faster for 4-IQBA. The k_{on} trend for all boronic acids is in the following order: D-fructose > D-tagatose > D-mannose > D-glucose. For example, k_{on} for D-fructose is 287 M^{-1} s^{-1} and only 0.6 M^{-1} s^{-1} for D-glucose upon binding with 8-IQBA. 5-IQBA and 4-IQBA also have the same tendency though the magnitudes are different. This observed trend in k_{on} is the same as the thermodynamic binding constants as described previously.[4] The various factors that affect the binding affinity between a boronic acid and a diol have been discussed elsewhere.[7,8]

In order to determine the thermodynamic binding constants, the k_{off} values were necessary. However, there is no good competitive reagent for the competitive binding with boronic acids. The k_{off} values were obtained directly from formula (1) by using the same data as k_{on}. To our surprise, the k_{off} values were all similar (Table 1). Such results are consistent with the fact that the trend for k_{on} rates generally parallels the known thermodynamic binding constant trend for the different sugar–boronic acid pairs. The only exception in the k_{off} rate was with D-glucose, which has a significantly lower k_{off} value than the other sugars. One possibility could be due to a known fact that boronic acid–glucose complexation goes through a structural change with time. Boronic acids are known to initially bind to D-glucose in the pyranose form, which then undergoes changes to the furanose form.[11,27–29] If the reverse follows the same pathway then one can understand why the dissociation is slower with D-glucose. With both the k_{off} and k_{on} rates on hand, we calculated the thermodynamic binding constants and the results are included in Table 1.

One thing that stands out from these kinetic data is that the K_a values obtained from the stopped-flow experiments are somewhat different from that obtained using the fluorescent method described earlier.[7,8,21,30] For example, for the binding between D-fructose and 8-IQBA, the stopped-flow kinetics experiments gave a k_{on} of 287 M^{-1} s^{-1} and a k_{off} of 0.38 s^{-1}, resulting in an observed K_a of 755 M^{-1}, while fluorescent tests gave a K_a 1493 M^{-1} between 8-IQBA and D-fructose. This is a 2-fold difference. K_a between 8-IQBA and D-glucose was determined to be 46 M^{-1} using a fluorescence method, whereas K_a is 4.6 M^{-1} using the stopped-flow experiment with a k_{on} of 0.6 M^{-1} s^{-1} and k_{off} of 0.13 s^{-1}. This is a 10-fold difference. 5-IQBA and 4-IQBA have even larger variations compared to 8-IQBA and variations of boronic acids with different sugars lack obvious tendencies. For example, K_a for the binding between 5-IQBA and D-fructose using stopped-flow kinetics was determined to be 209 M^{-1} with a k_{on} of 44 M^{-1} s^{-1} and k_{off} of 0.21 s^{-1}. However, fluorescence tests gave a K_a of 1432 M^{-1} for the 5-IQBA and D-fructose pair. This is a 7-fold difference. For the K_a between 5-IQBA with D-tagatose, D-mannose and D-glucose, there are 8-, 1- and 7-fold differences between fluorescence and stopped-flow kinetics techniques. The largest difference is the binding between 4-IQBA and D-tagatose with observed K_a 1651 M^{-1} from fluorescence tests and 167 M^{-1} using stopped-flow kinetics.

From the above studies, several things are clear. First, the binding process is generally fast, reaching equilibrium in seconds to minutes, depending on the boronic acid-sugar pair and the concentration. In the slowest case studied (e.g., 4-IQBA with glucose), it could take about 80 min to reach equilibrium at 1 mM concentration of the sugar-boronic acid pair. Second, the varying binding constants with different boronic acid-sugar pairs largely arise from the difference in the 'on' rate. Third, understandably the trend in k_{on} rates follows that for the thermodynamic binding constants for various sugars. Fourth, there is some numerical discrepancy in the binding constants calculated from the kinetic data as compared with those obtained using fluorescence. It is important to note that the magnitude of difference is greatest with those with the lowest binding constants, such as D-glucose. Such results can be correlated with the known phenomena that boronic acid first

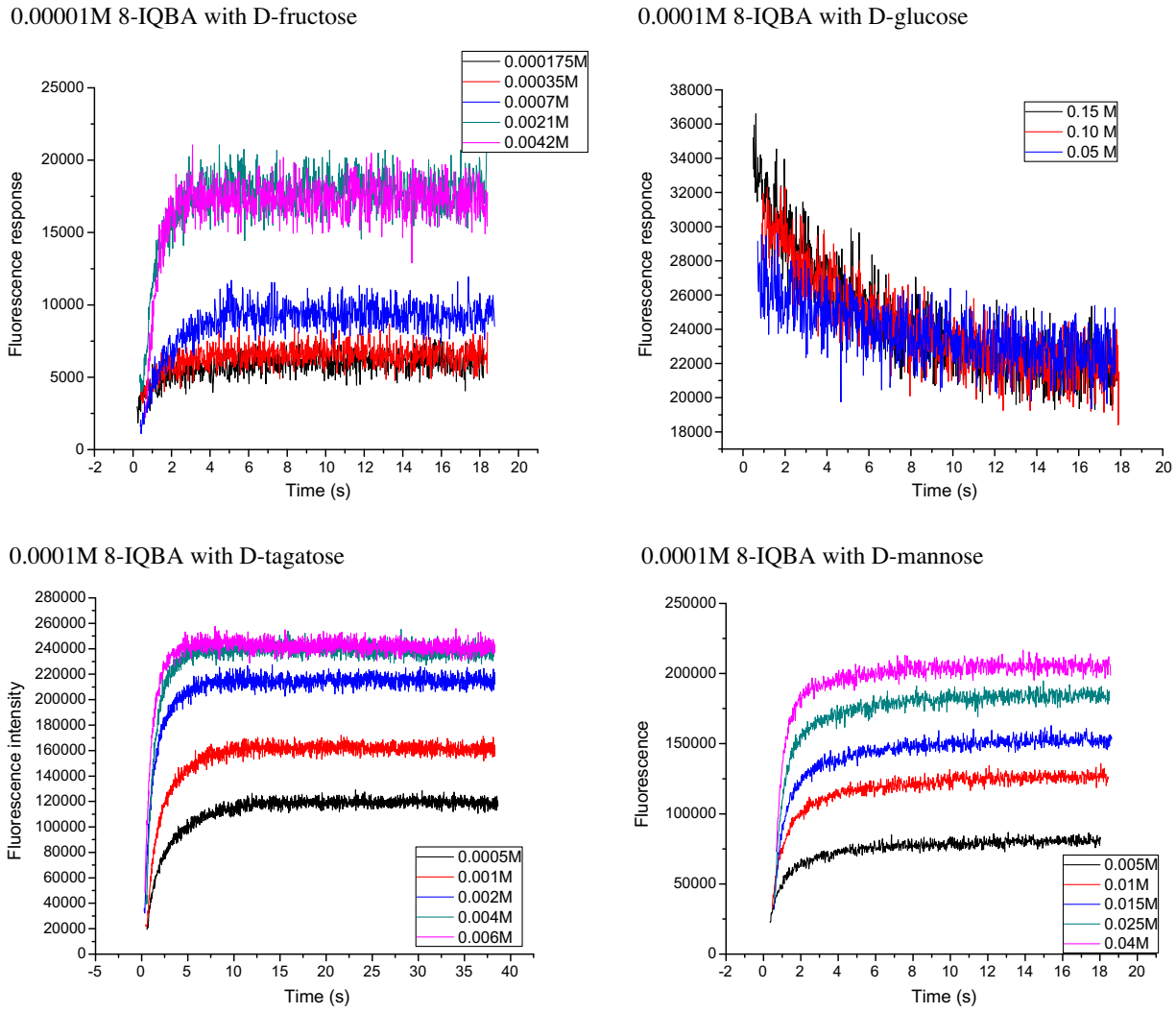


Figure 2. Fluorescent intensity changes of 8-IQBA at different time upon addition of sugars in phosphate buffer (0.1 M) at pH 7.4: λ_{ex} = 322 nm, λ_{em} = 383 nm (for D-fructose), λ_{ex} = 322 nm, λ_{em} = 382 nm (for D-tagatose), λ_{ex} = 322 nm, λ_{em} = 386 nm (for D-mannose) and λ_{ex} = 322 nm, λ_{em} = 361 nm (for D-glucose).

binds to glucose in the pyranose form and then slowly change to the more stable furanose form. Thus, the stopped-flow method determines the binding constant of the initial complex, while the thermodynamic fluorescent method determines the more stable furanose complex. In addition, many saccharides used in the study can exist in multiple forms in aqueous solution. Thermodynamic studies, which allow equilibrium time of over 10 min, give enough time for inter-conversion among different forms, which would affect the apparent binding constant. On the other hand, the stopped-flow method may just take a snap shot of the 'equilibrium' without actually allow for the different forms of the sugar to re-equilibrate. Of course, there may be other reasons for the observed discrepancy including the possibility of systematic experimental errors in determining the thermodynamic binding constants when the sugar concentration becomes too high, which could affect the viscosity and general property of the solution, and thus fluorescent properties of the fluorophore. The other possibility could be because of the somewhat different experimental conditions used. There may even be other factors due to the fact that the instrument used has a dead time of over 50 ms. Thus, early events that could affect the numerical calculations of 'on' or 'off' rates may have been overlooked.

There are a few other observations that should also be noted. Firstly, it is interesting that 4-IQBA showed fluorescent intensity decreased upon binding with D-glucose in the stopped-flow instrument, while we observed fluorescent intensity increased in the binding study using the fluorometer as reported before.[4] One possible reason is again associated with the structural change of the complex. It is possible that the species observed in the stopped-flow experiments was in the pyranose form, while the thermodynamic experiments observed the furanose form. Secondly, the stopped-flow signal for 5-IQBA with D-mannose is also abnormal. In the stopped-flow instrument, Figure 1 (Supplementary data) clearly indicates that binding is accompanied by an observed fluorescence increase. However, the fluorescence intensity decreased when we performed binding study using a fluorometer. One reason is again related to the possible formation of two species after complexation. However, at this time we have no evidence of what that might be.

4. Conclusions

In conclusion, we have described a series of stopped-flow studies of the binding between isoquinolinylboronic acids with

different sugars. With all the boronic acid–diol pairs studied, the reactions were complete within seconds under stopped-flow conditions. However, if the reactions were conducted at a dilute condition, the situation could be very different. Some reaction may take over 80 min to reach equilibrium at 1 mM concentration. All of the boronic acids have different k_{on} rates with the following order: D-fructose > D-tagatose > D-mannose > D-glucose. The k_{on} reaction rate is the key factor in determining the binding constant. There is some discrepancy in the numerical binding constants, which might be attributed to various reasons. For the examination of the detailed kinetic steps, a faster instrument would be needed. Such studies are reserved for future work. Our overall goal of understanding the reaction time scale has been achieved.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.014.

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