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A Competition Assay for Determining Glucose-6-phosphate Concentration with a *Tris*-boronic Acid Receptor

Larry A. Cabell, Mary-Katherine Monahan, and Eric V. Anslyn*

*Department of Chemistry and Biochemistry
The University of Texas at Austin
Austin, TX 78712

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Abstract: A *tris*-boronic acid receptor selective for glucose-6-phosphate is employed along with 5-carboxyfluorescein as an indicator in a competitive spectrophotometric assay to determine glucose-6-phosphate concentrations in the micromolar range.

Glycolysis is the major biological pathway for the generation of metabolic energy.[1] The first step in glycolysis is the conversion of glucose to glucose-6-phosphate.[2] Detecting the presence and concentration of glucose-6-phosphate by non-enzyme based sensors would be a valuable medical tool and would contribute to the science of sugar recognition.

In 1953, Föster described a competitive method for determining association constants involving a series of optical measurements.[3] This simple method for determining association constants avoids certain conditions that must be obeyed under a Benesi-Hildebrand analysis.[4] Another advantage innate to this competitive method is the presence of an indicator or a surrogate substrate. In the event that binding of the analyte and the receptor does not produce a significant spectral change, the equilibrium established between an indicator and a receptor will produce a spectrophotometric response. Once equilibrium has been established, introduction of the analyte to the indicator-receptor ensemble will result in a change of the equilibrium of the indicator-receptor ensemble relative to the association constants of the analyte-receptor and indicator-receptor complexes.[5] Following this concept, we have developed competition assays involving small synthetic molecules (receptors), not only to determine association constants between analytes and receptors, but also as sensing systems.[6] This novel use of a competition assay converts synthetic receptors into sensors without introducing additional covalent architecture. Herein, we report a competition assay based on the spectrophotometric observation of a 5-carboxyfluorescein-receptor complex and its conversion to a glucose-6-phosphate-receptor complex. The binding of glucose-6-phosphate with the receptor displaces the indicator from the receptor, providing a spectrophotometric change in absorbance intensity, which serves as a signal transduction mechanism for presence of glucose-6-phosphate binding to our receptor.

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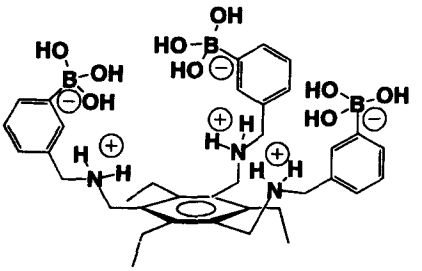
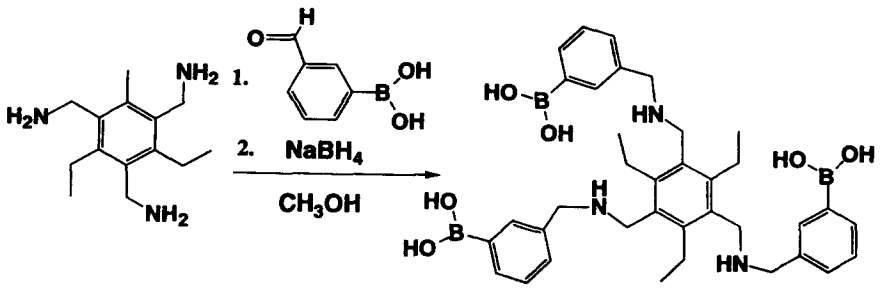
We have synthesized **1**, which positions boronic acid groups complementary to the hydroxyls of glucose-6-phosphate. In addition, three protonated secondary amines are positioned to coordinate the phosphate by electrostatic attractions. The boronic acids are positioned to coordinate the diol moieties through reversible covalent bond formation.[7] Preorganization of **1** is accomplished by incorporating the recognition groups into a 1,3,5-triethyl-2,4,6-trimethylbenzene scaffold. The alternating steric bulk around this C-3 symmetric spacer is preferred by approximately 3.5 *K*cal mol^{-1} in similar systems.[8]



Receptor **1**

Receptor **1**[9] was synthesized by the reaction of 1,3,5-*tris*-aminomethyl-2,4,6-triethylbenzene[10] with 3-formylbenzeneboronic acid under reducing conditions.



Scheme 1: Synthesis of **1**

Complexation of **1** with glucose-6-phosphate was studied using ^{1}H NMR, ^{31}P NMR and UV-vis spectroscopy. When the ^{31}P NMR spectrum of glucose-6-phosphate was followed with incremental increases in the concentration of **1** the data fit a 1:1 binding isotherm.[11] All binding studies were performed in a 70%

methanol / 30% deionized water solution by volume buffered with 4.0 x 10^{-2} M HEPES at a pH of 7.4. Computer fitting of the experimental isotherm with a typical 1:1 binding algorithm produced a binding constant of 1.6 x 10^3 M^{-1}.[12] The binding of **1** with 5-carboxyfluorescein was studied using UV-vis spectroscopy. The absorbance spectrum of 5-carboxyfluorescein was followed with incremental increases in the concentration of **1**, up to a 100-fold excess of the 5-carboxyfluorescein concentration. Under Benesi-Hildebrand conditions, the 5-carboxyfluorescein-**1** binding constant was determined to be 3 x 10^2 M^{-1}.

After establishing the binding constants for glucose-6-phosphate and **1** as well as 5-carboxyfluorescein and **1**, experimental conditions were determined for a competition assay involving glucose-6-phosphate, **1**, and 5-carboxyfluorescein. The addition of **1** to the solution of 5-carboxyfluorescein resulted in an increase in the absorbance intensity at 494 nm due to 5-carboxyfluorescein-**1** binding (see Figure 1). The absorbance spectra of a solution containing 3.0 x 10^{-5} M 5-carboxyfluorescein and 3.0 x 10^{-4} M **1** was followed at 494 nm with incremental increases in the concentration of glucose-6-phosphate. Addition of glucose-6-phosphate decreased the absorbance intensity at 494 nm due to a shift in the 5-carboxyfluorescein-**1** equilibrium, until the absorbance spectrum approached the absorbance spectra of free 5-carboxyfluorescein (see Figure 1). Using the algorithm for competitive binding, an association constant of 2.2 x 10^3 M^{-1} was determined for glucose-6-phosphate and **1**, in good agreement with the value obtained using NMR (see above).

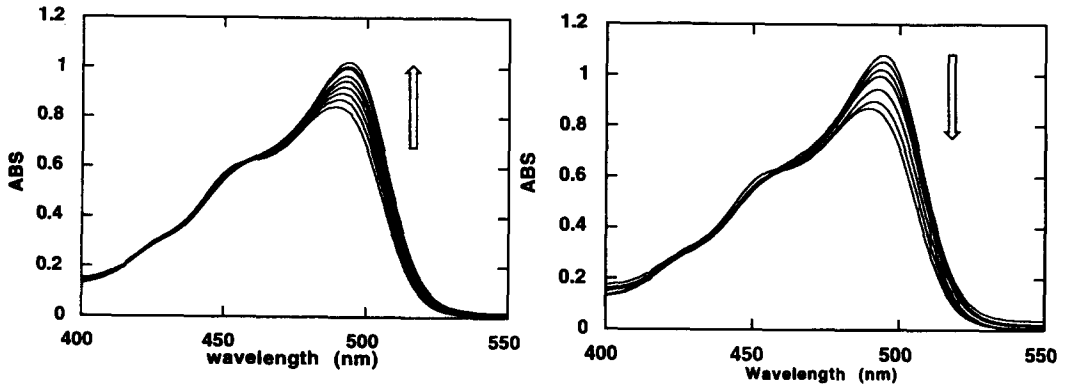


Figure 1: (Left) Titration of 5-carboxyfluorescein with incremental increases in the concentration of **1**. (Right) Titration of 5-carboxyfluorescein-**1** complex with incremental increases in the concentration of glucose-6-phosphate (Arrows indicate direction of change in the absorbance intensity).

Following the above procedure, the absorbance spectrum of 5-carboxyfluorescein and **1** was followed with incremental increases in the concentration of glucose up to a 100-fold excess over 5-carboxyfluorescein.

The glucose additions produced no detectable change in the 5-carboxyfluorescein-1 equilibrium. Additionally, the absorbance spectrum of 5-carboxyfluorescein and 1 was followed with incremental increases in the concentration of sodium phosphate up to a 100-fold excess also. The sodium phosphate additions produced no detectable change in the 5-carboxyfluorescein-1 equilibrium.

In conclusion, these studies demonstrated that receptor 1 discriminated well between glucose-6-phosphate and glucose or phosphate buffer. A competition assay involving 5-carboxyfluorescein, compound 1, and glucose-6-phosphate established a signal transduction mechanism observable by spectrophotometric techniques that allowed sensitive detection of the binding event between 1 and glucose-6-phosphate. Additionally, our results further confirm that competition assays give us an opportunity to extend the usefulness of synthetic receptors without introducing additional covalent bond architecture.

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[9] mp >250 °C (dec); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.80 (s, 3 H0, 7.73 (d, 3 H, J = 7.2 Hz), 7.49 (d, 3 H, J = 7.2 Hz), 7.39 (t, 3H, J = 10.4 Hz), 4.17 (s, 6H) 3.84 (s, 6H) 2.37 (q, 6H, J = 6.60 Hz), 0.77 (t, 9H, J = 6.9 Hz). ^{13}C {^{1}H} NMR (75MHz, CD_3OD) 144.4, 136.7, 135.2, 133.7, 133.4, 133.2, 127.8, 55.7, 46.3, 23.7, 17.0; CIHRMS m/z 652.392 (M^+ - H, $C_{36}H_{49}B_3N_3O_6$ calcd. Found 652.390).

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