Estimation of binding constants for the substrate and activator of *Rhodobacter sphaeroides* adenosine 5′-diphosphate-glucose pyrophosphorylase using affinity capillary electrophoresis

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Abstract

Binding constants were determined for the activator fructose-6-phosphate (F6P) and substrate adenosine 5′-triphosphate (ATP) (in the presence and absence of F6P) to the recombinant wild-type (WT) *Rhodobacter sphaeroides* adenosine 5′-diphosphate-(ADP)-glucose pyrophosphorylase (ADPGlc PPase) using affinity capillary electrophoresis (ACE). In these binding studies, the capillary is initially injected with a plug of sample containing ADPGlc PPase and noninteracting standards. The sample is then subjected to increasing concentrations of F6P or ATP in the running buffer and electrophoresed. Analysis of the change in the migration times of ADPGlc PPase, relative to those of the noninteracting standards, as a function of the varying concentration of F6P or ATP yields a binding constant. The values obtained were in good agreement with kinetic parameters obtained from steady state activity assays. The method was extended to examine the F6P binding constants for the R33A and R22A enzymes and the ATP binding constants for the R8A enzyme in the presence and absence of F6P. The R33A enzyme has been shown by activity assays to be insensitive to F6P activation, indicating a defect in binding or in downstream transmission of the allosteric signal required for full activation. ACE indicated no apparent binding of F6P, supporting the former hypothesis. The R22A enzyme was shown by activity assays to have a ~15-fold decrease in apparent affinity for F6P compared to that of WT while ACE indicated an affinity comparable to that of WT; potential reasons for this discrepancy are discussed. The R8A enzyme as measured by activity assays exhibits reduced fold-activation by F6P compared to that of WT but increased apparent affinity for ATP in the presence of F6P. The ACE results were in good agreement with the activity assay data, confirming the increased affinity for ATP in the presence of F6P. This method demonstrates the quantitative ability of ACE to study different binding sites/ligand interactions in allosteric enzymes.

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ADP-glucose pyrophosphorylase (ADPGlc PPase; EC 2.7.7.27) catalyzes the conversion of glucose-1-phosphate (Glc-1-P) and ATP to ADPGlc and pyrophosphate and is the rate-limiting enzyme in the production of biodegradable carbon sources such as glycogen and starch [1,2]. The regulation of bacterial ADPGlc PPase is mediated by the binding and effect of a number of metabolites which serve as either activators (glycolytic intermediates) or inhibitors (AMP, ADP, P_i) depending on the carbon pathway of the organism. These metabolites generally have an effect on both the V_max of the reaction and the apparent affinities for the substrates [1,2].

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Perhaps the most highly regulated ADPGlc PPases is from *Rhodobacter sphaeroides* which is activated by F6P, fructose-1,6-bisphosphonate (FBP), and pyruvate [3]. Although the importance of bacterial ADPGlc PPases in plant engineering and agriculture are well known [4,5], only enzymes from four of the nine defined regulatory classes [1] (represented by *Escherichia coli*, *Anabaena PCC 7120*, potato enzyme subunits, and *Agrobacterium tumefaciens*) have been studied in much molecular detail. For example, both chemical modification and site-directed mutagenesis studies of the *E. coli* enzyme have indicated the importance of Lys-39, Lys-195, and Tyr-[12]. Recent random mutagenesis and DNA shuffling approaches have revealed that several residues in both the N and the C terminus are responsible for binding and effect of the activator 3-PGA [9,10] while a conserved arginine plays a role in phosphate inhibition [11]. An alanine-scanning mutagenesis study of the *A. tumefaciens* enzyme revealed that specific arginines in the N terminus were responsible for interactions with the activators F6P and pyruvate [12]. Recent random mutagenesis and DNA shuffling approaches have revealed that several residues in both the N and the C termina of potato subunits of ADPGlc PPase are important in regulation [13,14]. The recent availability of the recombinant enzyme from the uniquely regulated *R. sphaeroides* enzyme [15] has opened up the possibility of further mutagenesis to better define the amino acids involved in regulation.

Numerous questions about the molecular basis of regulation for this enzyme family still need to be answered. In many cases, the effect of a mutation is desensitization to one or more effector molecules [9–12] as measured by the commonly used steady state kinetic activity assay [16]. Such a result leaves open the possibilities of loss of binding of the ligand or loss of postbinding communication of the allosteric signal. Very little is known about the actual binding of ligands to any ADPGlc PPase beyond an early study on the native *E. coli* enzyme [17] using the technique of equilibrium dialysis. This fairly time-consuming approach required the use of a relatively large amount of enzyme and radioactively labeled ligands. To further investigate binding to different forms of ADPGlc PPase, an additional analytical technique was warranted.

The powerful combination of separation and molecular characterization that is possible by affinity capillary electrophoresis (ACE) has made it possible to study many interactions and to estimate binding parameters [18–29]. The technique uses the resolving power of CE to distinguish between free and bound forms of a receptor/enzyme binding site as a function of the concentration of free ligand. A number of interactions providing information on binding parameters have been examined by ACE. For example, Zhang and Gomez [18] has demonstrated a multiple-step ligand injection approach using ACE to determine binding constants of ligands to receptors. Novotny et al. [19] have used ACE to investigate the interactions of concanavalin A with various saccharide oligomers. Finally, De Lorenzi et al. [20] used ACE to characterize the interaction between drugs and transferrin. In ACE the amount of free and bound receptor need not be known since changes in the migration time of the receptor are used in the estimation of a binding constant.

In this study we demonstrated the use of ACE to determine binding constants of the substrate ATP and the major activator F6P for WT *R. sphaeroides* ADPGlc PPase and extended the technique to include three altered forms of the enzyme. In these studies a plug of enzyme and noninteracting markers are injected onto the capillary and electrophoresed in increasing concentration of F6P or ATP. Changes in the migration time of the enzyme as a function of F6P/ATP yield a value for $K_b$ which can be converted to a $K_d$ value for comparison to the $A_{0.5}$ and $S_{0.5}$ values determined by activity assays. This work represents the first use of ACE to probe the binding of native and altered ADPGlc PPases to diverse ligands.

### Materials and methods

#### Chemicals and reagents

All chemicals were analytical grade. 4-Carboxybenzenesulfonamide (4-CBSA) and *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid) (Hepes) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Vancomycin (Van) from *Streptomyces orientalis* and adenosine triphosphate, and F6P were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For the F6P studies, stock solutions (1 mg/ml) of 4-CBSA and vancomycin (3 mg/ml) were each prepared by dissolving the lyophilized proteins in buffer (48 mM Hepes, pH 7.5). For the ATP studies, stock solutions (4 mg/ml) of 4-CBSA and vancomycin (3 mg/ml) were each prepared by dissolving in buffer (50 mM Hepes, pH 7.5).

#### Apparatus

The CE system used in this experiment was a Beckman Model P/ACE 5500 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μm, a length from the inlet to detector of 37.5 cm, and a length from the detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold Software. The conditions used in CE were
as follows: voltage, 25 kV; current, 23 μA; detection, 200 nm; temperature, 23 ± 0.1°C.

Procedures

For the studies with F6P, a sample (3.6 nl) containing 4.29 mg/ml Van, 1.43 mg/ml 4-CBSA, 0.0027 mg/ml DMF in buffer solution, and 0.70 mg/ml ADPGlc PPase (recombinant WT, R33A, R22A) was pressure injected into the capillary for 3 s. The electrophoresis was carried out in a 48 mM Hepes buffer solution (pH 7.5) for 5 min and repeated at increasing concentrations of F6P (0–640 μM). Data sets were performed in at least triplicate with calculated $K_b$ values differing by a maximum of 10%.

For the studies with ATP, the final concentration of the ADPGlc PPases in the sample (WT, R8A) was also 0.70 mg/ml. The sample also contained 5 μl Van, 5 μl 4-CBSA, and 1 μl DMF in concentrations mentioned above along with enough buffer to total 35 μl per sample. The sample was then pressure injected into the capillary for 3 s. The electrophoresis was carried out in a 48 mM Hepes buffer solution (pH 7.5) and repeated at increasing concentrations of ATP (0–600 μM) in the presence and absence of 2 mM F6P. As indicated for F6P, runs were performed in triplicate and calculated $K_b$ differed by no more than 10%.

Expression of WT and altered ADPGlc PPases. The recombinant \textit{R. sphaeroides} gene was expressed from \textit{pIM4} expression vector \cite{15} in \textit{TOP10 E. coli} cells (grown in Terrific Broth) by induction of log phase cells (OD$_{600 nm}$ = ca. 0.5) with 1 mM isopropyl β-D-thiogalactoside. The induced cells were harvested after ca. 18 h of growth at 25°C by centrifugation at 2500 g and stored frozen at −20°C. Typical wet weight yields were 12–14 g/L and cells from 1 to 2 L were used for purification.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene). The following forward primers (and their complements) were used for conversion of arginine to alanine in the amino terminus \cite{(base changes are boldfaced)}:

- \textbf{R8A} 5’-CAA CCC CCA CTC GCA CTG ACC GCG-3’
- \textbf{R22A} 5’-GCC GGC GGC GCA GGC AGC CG-3’
- \textbf{R33A} 5’-G ACG GAC CGG GCA GGC AAA CCC-3’.

It should be noted that the GC-rich nature of \textit{R. sphaeroides} DNA often resulted in the inclusion of primer concatamers when using the mutagenesis kit under standard conditions. Optimizing both the primer annealing temperature (55–68°C) in the mutagenesis reaction and the inclusion of dimethyl sulfoxide (2–5%) were often necessary. The mutations and WT sequences were confirmed by DNA sequencing.

Purification of recombinant wild-type and altered ADPGlc PPases. All purification steps were carried out at 0–5°C (unless noted). The presence of enzyme was monitored in chromatography fractions by the pyrophosphorolysis assay \cite{30} as described below under ADPGlc PPase assay and kinetic analysis. Cells harboring the expressed wild-type or altered enzymes were resuspended (ca. 1 g cells/2 ml) in a buffer containing 50 mM glycyglycine, pH 7.5, 5 mM DTT, and 1 mM EDTA, disrupted by sonication, and then centrifuged at 12,000 g to obtain soluble cell extracts. The crude extract was heat treated in a water bath set at 60°C for 5 min in the presence of 30 mM K-phosphate (pH 7), cooled on ice, and then centrifuged at 12,000 g for 15 min. The purification procedure was modified from our previously published protocol \cite{15} to include phenyl Sepharose, UNO6 (Bio-Rad), and Blue A (Amicon) chromatography columns in place of AMP Sepharose and Superdex-200 size-exclusion columns. It was desirable to eliminate the AMP affinity column as we anticipated that binding to the column (via the allosteric site) could be compromised with some of the altered proteins. The heat-treated supernatant was filtered through Whatman 3MM paper in a Buchner funnel to remove trace particulates and loaded onto a Fractogel DEAE-650 “Tentacle” (45–90 μm, EM Separations) column (2.5 x 15 cm) that had been equilibrated with 15 mM K-phosphate (pH 7.5), 50 mM KCl, and 0.5 mM DTE (Buffer A). After washing with 3 column volumes of Buffer A, the enzyme was eluted from the column using Buffer B (150 mM K-phosphate (pH 7), 200 mM KCl, 0.5 mM DTE) by a half-column-volume gradient of 0–37% Buffer B followed by a 2-column-volume wash with 37% Buffer B and a final 3-column-volume gradient of 37–100% Buffer B. Active fractions were pooled and concentrated using Centriprep-30 ultrafiltration concentrators (Amicon). A 2 M solution of K-phosphate (pH 7.5) was added to the concentrated post-DEAE enzyme to raise the phosphate concentration to 1.2 M prior to loading onto a Phenyl Sepharose 6 (Fast Flow) column (2.5 x 8 cm, Pharmacia) equilibrated in the same buffer. After washing with 3 column volumes of Buffer A1 (1.2 M K-phosphate (pH 7.5), 0.5 mM DTT) the column was subjected to a 0–60% Buffer B1 (50 mM K-phosphate (pH 7.5), 0.5 mM DTT) gradient wash over a half-column volume. The gradient was then held at 60% Buffer B1 for a half-column volume prior to enzyme elution with 3-column-volume gradient of 60–100% Buffer B1. Active fractions were pooled and concentrated as previously described, desalted using a Bio-Rad Econo-Pac 10DG column into Buffer A2 (25 mM Hepes (pH 7), 0.5 mM DTT), and filtered (0.2 μm) prior to loading onto a UNO6 column (Bio-Rad, equilibrated in the same buffer) attached to a DuoFlow BioLogic chromatography system (Bio-Rad). The enzyme was eluted from the column using the following procedure: after washing with 3 column volumes of Buffer A2, the column was washed with a 0–15% Buffer B2 (Buffer B2 included 25 mM Hepes [pH 7],
50 mM glycine, 0.5 mM DTT, and 800 mM KCl), 3-column-volume gradient followed by a 30-column-volume gradient of 15–35% Buffer B2 to elute the enzyme. The active fractions were concentrated and desalted (as previously described) into Buffer A3 (50 mM Hepes [pH 7.5], 10 mM Mg²⁺ [from MgCl₂], 0.5 mM DTT) prior to loading onto a Amicon Blue A column (2.5 x 8 cm) equilibrated in the same buffer. The enzyme was eluted by a 2-column-volume wash with Buffer A3 followed by a 3-column 0–100% gradient with Buffer B3 (50 mM Hepes [pH 7.5], 400 mM KCl, 0.5 mM DTT). The pooled enzyme fraction was desalted using a Bio-Rad EconoPac 10DG column into Buffer C containing 50 mM Hepes (pH 7.5) and 0.5 mM DTE, further concentrated to >1 mg/ml, and stored at −80°C. The enzyme was found to be stable for at least 3 months. The enzyme was stored in small aliquots as repeated freeze/thaw cycles were found to result in some enzyme precipitation.

**Gel electrophoresis.** Denaturing gel electrophoresis was performed using 10% polyacrylamide:bisacrylamide (29:1) gels in the presence of 0.1% SDS as previously described [3] with the Bio-Rad Mini-Protean II system. Protein was detected by staining with Coomassie brilliant blue (R250, Sigma). Post-Blue A WT and altered enzymes were found to be at least 95% homogeneous by SDS–PAGE.

**ADPGlc PPase assay and kinetic analysis.** Enzyme assays performed in the pyrophosphorolysis direction [30] during purification included 80 mM Tris–Cl (pH 8.0), 0.4 mg/ml BSA, 8 mM Mg²⁺ (from MgCl₂), 5 mM NaF, 1.2 mM ADPGlc, 2 mM ³²Pp (500–2000 cpm/ nmol, NEN-DuPont), and 2 mM F6P in a final volume of 250 μl. Assays were performed at different enzyme concentrations to ensure steady state conditions. The assays were initiated by the addition of enzyme (typically 0.001–0.1 μg in 10 μl freshly diluted in 50 mM Hepes [pH 7.5] with 0.5 mg/ml BSA and 0.5 mM DTE). A unit of activity is defined as the amount of enzyme catalyzing the production of 1 μmol of [³²P]ATP per minute at 37°C.

The highly pure post-Blue A column enzyme was suitable for kinetic assays run in the ADPGlc synthesis direction [16]. Assays were initiated with the addition of enzyme (in 10 μl, diluted as described above, yielding ca. 2–20 nmol of product per assay). Depending on the protein concentration and assay conditions, typical enzyme dilutions used ranged from 200- to 10,000-fold. In all cases, enzyme dilutions assayed were in a linear range (enzyme concentration versus rate) during the time course of the assay. Data points typically represent the average of at least two determinations that differed by less than 10%. Standard assays for activity measurements, in the ADP-glucose synthesis direction, included 100 mM Hepes (pH 7.0), 0.5 mg/ml BSA, 1 mM [¹⁴C]Glc-1P (1000–2000 cpm/nmol, NEN-DuPont), 5 mM Mg²⁺, 1 mM ATP, and 0.2 U of inorganic pyrophosphatase (Sigma) and water in a total volume of 200 μl. Saturation plots for substrates and effectors were analyzed with the use of a computer program [31] using the Levenberg–Marquardt algorithm for regression. The S₀.₅ and A₀.₅ values are defined here as the concentrations of substrate and activator that give 50% maximal activity and activation, respectively, with all other ligands fixed at saturating concentrations.

**Results and discussion**

**Binding of F6P to ADPGlc PPases**

Prior to collecting data, optimal resolution of free and ligand-bound forms of the enzyme were determined to be at pH 7.5 in the described Hepes buffer in the absence of Mg²⁺. This simple system for measuring binding was considered to be relevant in that these conditions were essentially identical to those used in successful protection studies with F6P and ATP which prevented modification by the arginyl reagent butanedione [3].

In our first series of experiments we examined the binding of the activator F6P to recombinant *R. sphaeroides* WT ADPGlc PPase and two site-directed mutants (R33A and R22A). In this technique a sample containing Van, DMF, and 4-CBSA was injected into the capillary by pressure and electrophoresed in a solution of F6P for 5.0 min. Fig. 1 shows a representative series of electropherograms for F6P and WT ADPGlc PPase generated using the ACE technique. Upon addition of increasing concentrations of F6P in the running buffer, the ADPGlc PPase peak shifts to the right for all concentrations of F6P with respect to the noninteracting markers. DMF and Van are used as neutral and positively charged markers, respectively, and do not interact with F6P in the running buffer under the conditions of electrophoresis. 4-CBSA is a negative marker and does not interact with F6P in the running buffer under electrophoretic conditions. The binding of F6P to ADPGlc PPase results in an increasing negative charge on the enzyme and the ADPGlc PPase-F6P complex peak shifts to a longer migration time, with respect to the noninteracting standards.

**Fig. 2** is a Scatchard plot of the data for the recombinant *R. sphaeroides* WT ADPGlc PPase. In experiments employing two or more noninteracting standards, a relationship relating two of the standards to the receptor in question can be derived. We term this relationship the relative migration time ratio (RMTR)

$$ \text{RMTR} = \frac{t_r - t'_s}{(t'_s - t_s)} .$$

Here, $t_r$, $t_s$, and $t'_s$ are the measured migration times of the receptor (enzyme) peak and the two noninteracting standard peaks, respectively. A Scatchard plot can be obtained using Eq. (2):
Here, $\Delta \text{RMTR}_{R,L}/[L] = K_b \Delta \text{RMTR}_{R,L}^{\max} - K_b \Delta \text{RMTR}_{R,L}$.  

$$\Delta \text{RMTR}_{R,L}/[L] = K_b \Delta \text{RMTR}_{R,L}^{\max} - K_b \Delta \text{RMTR}_{R,L}. \quad (2)$$

Fig. 1. Representative set of electropherograms of WT ADPglc PPase in 48 mM Hepes buffer (pH 7.5) containing various concentrations of F6P using ACE. The total analysis time in each experiment was 5.0 min at 25 kV (current: 23 µA) using a 37.5-cm (inlet to detector), 50-µm-I.D. open, uncoated quartz capillary. Van, DMF, and 4-CBSA were used as internal noninteracting standards.
apparent binding of F6P to the R33A enzyme. It should be noted that the $V_{\text{max}}$ values in the absence of F6P for the WT, R22A, and R33A enzymes were 18.1 $\pm$ 0.5, 1.3 $\pm$ 0.2, and 118.5 $\pm$ 5 U/mg, respectively. The R33A enzyme was thus substantially active in the absence of F6P and insensitive to F6P. In contrast, the R22A enzyme has very low activity in the absence of F6P. The fold-activation by saturating F6P was found to be 8.3- and 57.8-fold to give $V_{\text{max}}$ values of 150.2 and 75.2 $\pm$ 2.9 U/mg, respectively, for the WT and R22A enzymes. With respect to response to F6P, the R33A and R22A enzymes are similar to the analogous altered enzymes from A. tumefaciens [12]. Briefly, R33 is in a relatively conserved region (amino acids 32–38 in R. sphaeroides, RRAKPAV) that has been associated with activator binding and/or effect [6,12,15]. The high concentration of positive charge could serve as part of an anionic binding site for activator. Arginine-22 is part of a relatively conserved glycine-rich region (amino acids 18–23 in R. sphaeroides, LAGGRG) that has been postulated to be involved in optimal substrate and activator binding and catalysis [1,12,15]. A detailed kinetic analysis of these and other R. sphaeroides enzymes generated by alanine scanning mutagenesis will be published elsewhere.

Table 1 compares the corresponding $K_d$ values from ACE to the $A_{0.5}$ values obtained for the WT [15] and altered proteins. The dissociation constant for the WT enzyme, as determined by ACE at pH 7.5, was in fairly good agreement with the $A_{0.5}$ value determined by activity assays at pH 7 (Table 1). It should be noted that the $A_{0.5}$ value determined at pH 8 was found to be 29.3 $\pm$ 2.4 $\mu$M [3], so the reported value at pH 7.5 is quite reasonable. Further, the absence of substrates and Mg$^{2+}$ in ACE appears to have little effect on F6P binding for the WT enzyme. It should be noted, however, that the binding stoichiometry between enzyme and F6P from the ACE data was estimated to be 1:1 as shown by linear Scatchard plots. A modified form of analysis using a 2:1 binding stoichiometry was a poor fit and was ruled out. Some cooperativity is generally observed (Hill Number $\approx$ 1.4–1.8) when fitting F6P saturation data from activity assays performed at saturating substrate concentrations. This apparent cooperativity may be due to the presence of substrates and Mg$^{2+}$ (which are not present in the binding assay). It has previously been hypothesized that ATP may be able to bind to the activator site as ATP and ATP/Mg protected the R. sphaeroides enzyme from F6P desensitization by chemical modification [3]. This is also consistent with equilibrium dialysis binding data with the E. coli enzyme that indicated that nonchelated ATP was competitive with the activator FBP [17].

The results with the R33A enzyme provide strong evidence for a defect in binding, as opposed to a downstream conformational effect, as being responsible for the kinetic behavior of the R33A enzyme. This is consistent with the chemical modification and protection data for the A. tumefaciens WT and R33A enzymes which supported a role for R33 in binding F6P. Interestingly, ACE indicates that the R22A enzyme has an affinity for F6P within 2-fold of the WT enzyme while the opposite result is found using activity assays which showed the R22A enzyme to have $\approx$15-fold lower apparent affinity for F6P than the WT enzyme. In this case, binding of F6P under the conditions of ACE appears to be even more favorable than that to WT. For this altered protein, binding appears to be uncoupled from the subsequent activation process. It should be noted that the change in apparent affinity for ATP is less affected than that for F6P, being only $\approx$4-fold lower than WT. As previously mentioned, there is evidence that ATP can bind to the activator site of ADPGlc PPase [3,17]. The result of this altered binding to the R22A enzyme is unproductive compared to WT, as previously mentioned, as the activity in the absence of F6P is less than 10% of WT.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_d$ (µM)$^a$</th>
<th>$A_{0.5}$ (µM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>21.3</td>
<td>119.0 $\pm$ 1.0</td>
</tr>
<tr>
<td>R22A</td>
<td>10.6</td>
<td>175 $\pm$ 18</td>
</tr>
<tr>
<td>R33A</td>
<td>—c —d</td>
<td>—c —d</td>
</tr>
</tbody>
</table>

$^a$ Reported values are the average from three experiments which differed by less than 10%. See Materials and methods for details.

$^b$ Estimate based on activity assay carried out at pH 7 in the presence of saturating concentrations of ATP and Mg$^{2+}$ as described under Materials and methods.

$^c$ No binding detected.

$^d$ No change in activity in response to increasing F6P concentration.

Fig. 2. Scatchard plot of the data for recombinant R. sphaeroides WT ADPGlc PPase.
Perhaps in the activity assay, the ATP and/or ATP/Mg present is binding more effectively to the activator site than F6P, requiring an increased concentration of F6P to displace ATP. Further binding studies are in progress for this interesting enzyme.

**Binding of ATP to ADPGlc PPases**

In a second series of experiments we examined the binding of ATP to WT and the R8A enzyme in the presence and absence of 2 mM F6P. In this series of experiments the protein and standards were injected into the capillary column and the concentration of ATP in the running buffer sequentially increased. Fig. 3 shows a representative series of electropherograms for ATP and WT ADPGlc PPase. A trend in the movement of the protein peak similar to that found in the binding of F6P to the enzyme is observed. Fig. 4 is a Scatchard plot of the data. The $K_b$ values ($10^3$ M$^{-1}$) for the WT enzyme in the presence and absence of 2 mM F6P were determined to be 20.8 and 5.1, respectively. For the R8A enzyme, these values were found to be 12.5 and 5.3. It should be noted...
that the R8A enzyme has an affinity for F6P very similar to that of the WT enzyme. A major difference from WT is that the R8A enzyme exhibits a ~6-fold higher $V_{\text{max}}$ (105.6 ± 3.5 U/mg) in the absence of F6P. The presence of F6P activates the R8A enzyme only 1.4-fold to an activated $V_{\text{max}}$ similar to WT. As indicated earlier, a detailed kinetic analysis of the R8A and other altered enzymes will be published elsewhere.

Table 2 provides the calculated $K_d$ values from ACE along with the $S_{0.5}$ values from the fitting of the activity assay data for the WT and R8A enzymes. The ACE data are in very good agreement with the enzyme assay data, indicating that the $S_{0.5}$ values reflect actual binding. The increased affinity for ATP at the active site in the presence of F6P is seen as the K-type component of activation in activity assays. This is clearly independent of the $V_{\text{max}}$ effects of the mutation on the enzyme.

## Conclusion

ACE was successfully used to determine binding constants of F6P and ATP in the presence and absence of F6P to recombinant *R. sphaeroides* WT ADPGlc PPase. The resulting $K_d$ values were in good agreement with the $A_{0.5}$ and $S_{0.5}$ values obtained from steady state kinetic activity assays. ACE was also used to estimate binding of F6P to the activator-insensitive R33A enzyme and the R22A enzyme. For the R33A enzyme, the ACE results clearly indicated that the lack of response observed in the activity assay was due to a loss of F6P binding. For the R22A enzyme, the binding of F6P in the absence of ATP is similar to that of WT as measured by ACE. The altered effect of ATP and/or ATP/Mg appears to decrease the apparent affinity for F6P as measured in the activity assay. The $K_d$ values for the highly active R8A enzyme in the presence and absence of F6P were in good agreement with the determined $S_{0.5}$ values.

As previously indicated, the technique of equilibrium dialysis had been successfully used to estimate binding constants for ligands to the *E. coli* enzyme [17]. The advantage of using CE is that it requires small quantities of material, is highly sensitive, is very flexible, and has high resolution. This method demonstrates the quantitative ability of ACE to study different binding site interactions. This opens up the possibility of examining the binding of the other anionic activators and inhibitors of the *R. sphaeroides* and other ADPGlc PPases (WT and altered) alone and in combination to determine interactions between effectors at the various binding sites.

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