

## Microtiter assay for glutamine synthetase biosynthetic activity using inorganic phosphate detection

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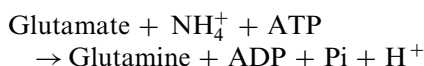
### Abstract

A microtiter assay was developed for the improved detection of inorganic phosphate released from adenosine 5'-triphosphate (ATP) in the glutamine synthetase biosynthetic assay. In this assay, ascorbic acid replaces the traditionally used ferrous sulfate to reduce the phosphomolybdate complex. As a result, increased color development, linearity, and sensitivity are achieved. Additionally, in the microtiter format, multiple sets of kinetic experiments can be rapidly performed in parallel. The color that forms is rendered highly stable by the addition of sodium citrate. However, the commonly used sodium arsenite in this solution has been omitted, making the assay less hazardous. The assay is linear to 100 nmol Pi in the presence of 10 mM ATP.

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Glutamine synthetase (GS)<sup>1</sup> (glutamate-ammonia ligase; EC 6.3.1.2) is a key enzyme in nitrogen metabolism and ammonium assimilation and catalyzes the reaction



in the presence of  $\text{Mn}^{2+}$  or  $\text{Mg}^{2+}$ . A variety of GS assays have been reported [1–4]. The most commonly used are the “transferase” and “forward” assays that rely on the formation of  $\gamma$ -glutamylhydroxamate from hydroxylamine and glutamine or glutamate, respectively [3,4]. These assays are convenient since they can be performed with crude extracts and in the presence of phosphatases. However, they do not measure the physiologically relevant reaction of GS shown above and are therefore unsuitable for determining kinetic parameters of the enzyme. To measure the enzyme activity more directly, a

continuous coupled assay and an assay based on the phosphate released from ATP have been developed [1–3,5]. In the continuous assay, the regeneration of hydrolyzed ATP is coupled to the oxidation of NADH in the presence of excess phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase [3,5]. In the phosphate release assay, the Pi released from ATP is measured using a colorimetric ammonium molybdate-based detection method [1]. This endpoint assay is less complicated to perform than the continuous assay but suffers from a lack of sensitivity [1].

The colorimetric determination of phosphate typically involves the interaction of phosphate and molybdate and reducing agents or dyes to produce a colored complex [6]. The reduction of the phosphomolybdate complex in a strong acid solution results in a formation of “molybdenum blue.” Numerous assays for quantitating phosphate based on this reaction have been developed with varying sensitivities and applications, mainly by altering the nature of reducing agents, since the early work of Bell and Doisy [7] and Fiske and Subbarow [8]. In the GS biosynthetic assay [1,2], the Pi detection technique used ferrous sulfate as the reducing agent [9]. In other Pi detection methods used for other purposes, color intensity and sensitivity were greatly increased

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<sup>1</sup> Abbreviations used: GS, glutamine synthetase; SDS, sodium dodecyl sulfate; Mops, 3-morpholinopropanesulfonic acid.

when ascorbic acid was used as the reducing agent [10,11]. Further improvements to these Pi detection methods were made when citrate was introduced to complex excess molybdate and consequently stop color development. Once stopped, additional color formation from the potential hydrolysis of labile organic phosphates was prevented [12–14].

Here we have adapted methods previously used for detecting Pi in the presence of high ATP concentrations to the GS biosynthetic assay [15,16]. The linear range of detection has been extended from less than 5 to 100 nmol orthophosphate per assay. This assay was highly sensitive and stable in the presence of 10 mM ATP. The development of this assay greatly increases the productivity and sensitivity of the GS assay, particularly when used in a microtiter plate format.

## Materials and methods

### Reagents and materials

The following solutions for phosphate detection were prepared with double-distilled water in 50-ml plastic tubes. All chemicals were purchased from Sigma–Aldrich Corp (St. Louis, MO): Solution A, 12% w/v L-ascorbic acid in 1 N HCl; Solution B, 2% w/v ammonium molybdate tetrahydrate in ddH<sub>2</sub>O; Solution C, 1% w/v ammonium molybdate tetrahydrate in ddH<sub>2</sub>O; Solution D, mixture of two parts Solution A and one part Solution B; Solution E, mixture of two parts Solution A and one part Solution C; Solution F, 2% sodium citrate tribasic dihydrate and 2% acetic acid in ddH<sub>2</sub>O; and phosphate standard, 20 mM potassium phosphate dibasic in ddH<sub>2</sub>O. Solutions A, B, and C were stored at 4 °C and were stable for 1 week. Solutions D and E were prepared just before use and were stable for 1 h at room temperature. Solution D was used when ATP was present at 10 mM in the reaction mix for the glutamate and NH<sub>4</sub>Cl enzyme kinetic assays. Solution E was used when ATP enzyme kinetics were studied (see Results and discussion). Solution F was stable at room temperature for 2 months. Phosphate standards were prepared and stored frozen at –20 °C as 1-ml aliquots in 1.5-ml microcentrifuge tubes.

### Assays

The GS biosynthetic reaction mix was adapted from Woolfolk et al. [2] and contained 100 mM Mops (pH 8.2), 50 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 250 mM monosodium glutamate, 50 mM NH<sub>4</sub>Cl, 10 mM ATP, and purified recombinant unadenylylated GS from *Alteromonas macleodii* isolated from an adenylation-deficient *Escherichia coli* strain. After addition of all components, the final pH of the reaction mix was adjusted to pH 7.5, which was the pH optimum for the enzyme determined using the

biosynthetic reaction. Sufficient GS was added to the reaction mix to produce between 35 and 50 nmol Pi in 5 min.

To determine the apparent kinetic parameters of *A. macleodii* GS, a series of dilutions for glutamate, ammonium, or ATP was prepared in 12-well reservoirs (USA Scientific, Ocala, FL). When one of the substrate sets was added to initiate the reactions, the other substrates remained at saturating concentrations indicated above. In a set of reactions, 90 µl of the assay/enzyme mix was added to 16 PCR tubes (two 8-tube strips; USA Scientific) using a multichannel pipettor and equilibrated for 5 min at the desired temperature (10–50 °C) in a 96-well-format thermal cycler (Eppendorf Mastercycler; Brinkmann Instruments, Westbury, NY). The reactions were initiated by adding 10 µl of the substrate dilution set with a multichannel pipettor. A set of phosphate standards ranging from 0 to 20 mM was also prepared; 10 µl of each dilution of the phosphate standard was added to 90 µl reaction mix instead of the initiation substrate. After 5 min, 50 µl of the reactions was removed with a multichannel pipettor and transferred to 150 µl Solution D or E, previously delivered to wells in a 96-well flat-bottomed Nunclon Microwell plate (Nunc, Roskilde, Denmark), mixed well, and held at room temperature. The low pH of the color reagent terminated the reaction. Exactly 5 min later, 150 µl Solution F was added to the wells to stop color development. Blanks (10 µl ddH<sub>2</sub>O added to 90 µl assay/enzyme mix) were included in one tube of the phosphate series and in the set of substrates. The reaction was allowed to equilibrate for 15 min at room temperature before reading the absorbance at 655 nm with a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Absorbance readings were input to Excel (Microsoft) and the least squares fit to the Michaelis–Menten or Hill equation was determined using the Solver in the Tools menu [17]. Alternatively, the kinetic parameters were determined using Kaleida-Graph (Synergy Software, Reading, PA).

## Results and discussion

Although numerous microtiter malachite green-based phosphate detection assays have been described [6,18–21], the narrow range of detection was not readily adaptable to the GS assay. Other detection methods with broader detection range and enhanced ATP stability have been developed for other enzymes based on the ascorbic acid reduction of the phosphomolybdate complex and were more applicable to the present study with GS [14–16,22]. To measure Pi released from ATP in the GS biosynthetic activity, modifications have been made to these latter detection assays [15,16] (Table 1). In our case, the SDS treatment step was not necessary to stop the GS reaction. The reactions contained low

Table 1  
Comparison of phosphate detection methods used in the presence of ATP

	Chifflet et al. [15] <sup>a</sup>	Gonzalez-Romo et al. [16] <sup>a</sup>	This study
Enzyme reaction volume (μl)	50 <sup>b</sup>	150 <sup>b</sup>	100 <sup>c</sup>
Range (nmol)	0–20	0–60	0–100
Ascorbic acid (%) <sup>d</sup>	1.5	3	6
Molybdate (%) <sup>d</sup>	0.25	0.5	0.25 or 0.5
HCl (N) <sup>d</sup>	0.25	0.25	0.5
Color development stop solution	2% Na-citrate, 2% Na-arsenite, 2% acetic acid	2% Na-citrate, 2% Na-arsenite, 2% acetic acid	2% Na-citrate, 2% acetic acid
Total volume (μl)	350	1050	350

<sup>a</sup> Assays developed for ATPase determinations.

<sup>b</sup> Reaction terminated by the addition of an equal volume of 12% SDS.

<sup>c</sup> Reaction terminated by transferring 50 μl of the GS reaction into the color reagent.

<sup>d</sup> Final concentrations during color formation reaction but before adding color development stop solution.

concentrations of purified GS and were terminated by direct transfer into the acidic color reagent. During color formation, ascorbic acid and HCl have been increased to 6% and 0.5 N, respectively. The amount of ammonium molybdate was 0.25 or 0.5% depending on conditions. Finally, sodium arsenite has been omitted from the color development stop solution (Solution F), without compromising the linearity of the assay (Table 1, Fig. 1).

The absorbance spectrum of the colored product of this assay was different from that previously reported. Instead of maxima at 700 and 850 nm [13,15], a single broad peak was identified at 815 nm (data not shown). This may be attributed to omission of sodium *meta*-arsenite in the citrate solution or to components of the reaction mix. Arsenite has been suggested to increase the color intensity of the phosphomolybdate complex by an unknown reaction [13]. However, arsenite does not appear to be necessary for the present assay, and, more importantly, the handling and disposal of this toxic compound is avoided. Phosphomolybdate assays are commonly read at 660, 700, or 850 nm [2,12,13,15,16]. In this assay, microtiter plates were read at 655 nm, and

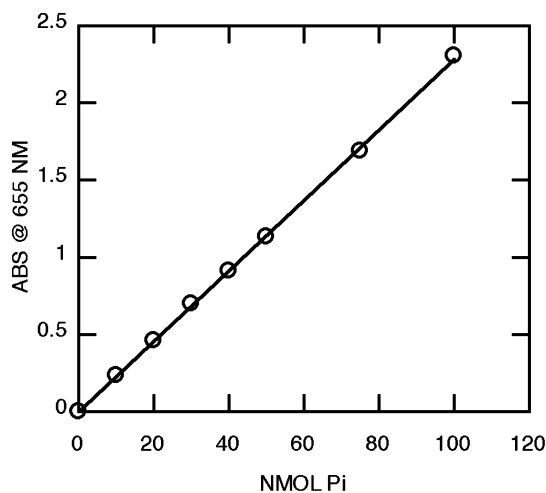


Fig. 1. Typical standard curve for inorganic phosphate added to GS reaction mix containing 10 mM ATP.

absorbance values were approximately 65% of the maximum absorbance at 815 nm (data not shown). The standard curve at 815 nm was useful only to 60 nmol Pi due to extremely high absorbance values. However, greater sensitivity at lower levels of phosphate could be achieved in this assay simply by reading at a higher wavelength.

Phosphate standards were incorporated into each microtiter plate of GS assays. The standard curves were highly reproducible and linear from 0 to 100 nmol Pi in the reaction volumes noted (Fig. 1). The slope of the standard curve was approximately 11 times greater than that achieved in the previous GS assay [2] and as a result sensitivity is greatly enhanced (data not shown). When the standard curve was prepared in reaction mix, the slight orthophosphate contamination in the ATP was corrected for by the reagent blank. The blue color developed within 1 to 2 min in reaction mix and was stable in the presence of 10 mM ATP (Fig. 2). In the GS

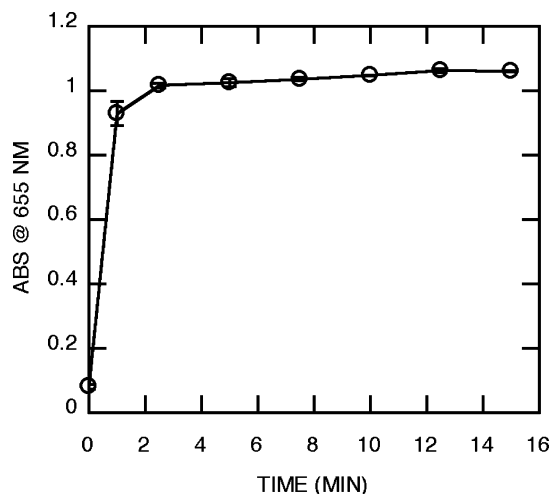


Fig. 2. Time course of color development. Samples (50 μl) containing 50 nmol Pi in GS reaction mix were added to 150 μl color reagent. The color formation was allowed to continue over the indicated time interval before addition of Solution F. The reaction mix contained 10 mM ATP (500 nmol ATP). No significant ATP hydrolysis was observed. The plot shows the average of two sets where the error bars indicate one SD.

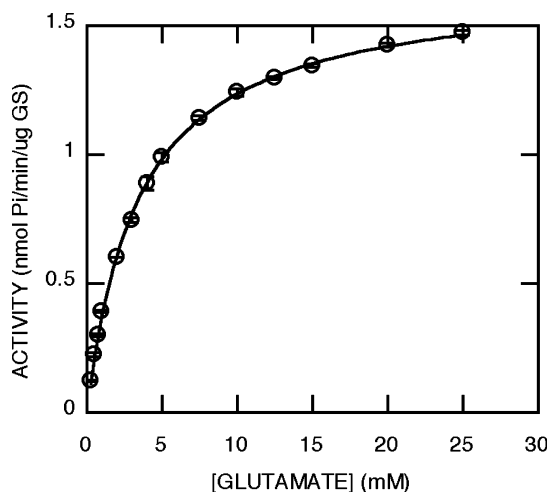


Fig. 3. Determination of kinetic parameters for *A. macleodii* GS for glutamate at 10 °C. The plot displays the average of two sets of reactions with error bars showing one standard deviation. The solid line represents the least squares fit of the Michaelis–Menten equation to the experimental data with the apparent  $K_m(\text{Glu}) = 3.49$  mM and  $V_{\max} = 1.67$  nmol Pi/min/ $\mu\text{g}$  GS.

assays, color development was terminated by the addition of citrate at 5 min, but it can be added at any time between 3 and 10 min (Fig. 2). Citrate complexes the free molybdate in solution and prevents further color formation from the possible hydrolysis of labile organic phosphates that might occur during color development [12,13]. The color was stable for at least 6 h (data not shown).

Molybdate has been noted to form complexes with organic phosphates including ATP but does not directly contribute to color formation [13]. As a result, additional molybdate is required in the presence of high ATP concentrations to maintain linearity of the standard curve [13]. This becomes important when saturating ATP levels are used for determining the kinetic parameters for glutamate or ammonium. In this case, the concentration of molybdate in the color reagent was increased using Solution D. However, when the kinetic parameters for ATP were determined, the ATP concentrations were varied from 0 to 5 mM and Solution E was used instead. This modification did not significantly change the slope of the standard curve. The average change in absorbance at 655 nm was  $0.023 \pm 0.0017$  U/nmol Pi using Solution D and  $0.022 \pm 0.0003$  U/nmol Pi using Solution E.

A Michaelis–Menten plot for deriving the  $K_m$  for glutamate for the GS from *A. macleodii* is shown in Fig. 3. The apparent  $K_m$  for glutamate was calculated at 3.49 mM and the  $V_{\max}$  was 1.67 nmol Pi/min/ $\mu\text{g}$  GS. The assays were performed in duplicate and were highly reproducible (Fig. 3). Likewise, kinetic parameters were accurately determined for ATP and  $\text{NH}_4\text{Cl}$  using this assay (data not shown). It is noteworthy to mention that

during the development of the assay procedure, GS reactions were initially incubated in microtiter plates instead of PCR tubes. However, the reactions equilibrated slowly and temperature gradients were observed across the plate, especially at higher temperatures. The thermal cycler set at the desired temperature enabled accurate temperature control between individual reactions and at different temperature settings from 10 to 50 °C. The use of the PCR tubes required an additional transfer step, but can be performed accurately (Fig. 3).

The GS biosynthetic assay described here has numerous advantages over previous methods [1,2]. Most importantly, the sensitivity for detecting phosphate has been greatly increased by using ascorbic acid as the reducing agent. By instituting a microtiter format, the initial velocities can be determined on a set of 16 reactions simultaneously and can be scaled up for additional reactions. Multiple sets or sets with different enzymes can be run at the same time, and absorbance readings can be rapidly determined with a plate reader. With the use of the thermal cycler, the reaction temperature is accurately maintained over a wide range. Finally, arsenite is not required in the citrate solution used in this assay and the color is stable for hours.

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