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ATP binding to purified homopolymeric plant glutamine synthetase studied by isothermal titration calorimetry

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Abstract

Adenosine-5′-triphosphate (ATP) binding to purified plant glutamine synthetase (GS) recombinantly overexpressed in *Escherichia coli* was investigated by enzyme kinetics and isothermal titration calorimetry (ITC). The concentrated enzyme was highly stable at ITC working conditions (25 °C). However, diluted preparations of the enzyme were considerably less stable but the addition of ethylene glycol to the buffer improved the long-term stability at 25 °C, although this compound precluded any possible microcalorimetric measurement. Thermodynamic parameters of binding of ATP to purified homopolymeric GS were determined both in Tris and Hepes buffer and at different ionic strength. Proton uptake by the protein was clearly detected upon ATP binding. The data obtained fitted better to a model with an n value of about one, suggesting that each enzyme subunit is able to bind a molecule of ATP. Data were also compatible with kinetic estimates of K_m. We think that this kind of approach will help the structure–function characterisation of plant GS by the comparative study of wild-type and site-directed mutant polypeptides.

Keywords: Glutamine synthetase; Isothermal titration calorimetry; ATP; Binding; Recombinant plant enzyme

1. Introduction

Nitrogen assimilation is a crucial process in terms of biological productivity particularly in plants [1] and it also has health and environmental implications. All forms of inorganic nitrogen absorbed by biological organisms have to be reduced to ammonium for their assimilation. Glutamine synthetase (GS; EC 6.3.1.2) plays a key role in nitrogen assimilation catalysing the incorporation of ammonium into glutamate to yield glutamine. The bacterial enzyme (GS type I) is a dodecamer composed of two face-to-face hexameric rings; the fine structure of this GS type was first determined by X-ray diffraction studies from crystals of *Salmonella typhimurium* enzyme [2]. A reaction model for the mechanism of bacterial GS has been proposed based on X-ray data from *Salmonella* GS crystals soaked with different substrates [3]. The presence in the amino acidic sequence of eukaryotic GS (octameric type II enzyme) of several residues corresponding to the active site of GS type I, lead to the proposal that a common reaction mechanism should be operating in the two types of enzyme [4]. In our laboratory, we have achieved the site-directed

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mutagenesis of the α-polypeptide of *Phaseolus vulgaris* type II GS (cytosolic isoform). The mutagenesis process was focused on residues that could correspond to active site residues in GS type I. Enzymological studies of these mutants polypeptides, recombinantly expressed in *Escherichia coli*, suggested that the catalytic role of some highly conserved residues of the plant enzyme are quite different from the bacterial ones [5,6], while other residues appeared to be functionally conserved [7]. In the absence of high-resolution structural information for eukaryotic GS, microcalorimetry is a good technique for investigating the thermodynamic of substrate binding to the active site and, by comparing data from the wild-type and mutant proteins, structure–function information can be extrapolated. The prokaryotic (type I) GS was analysed by microcalorimetric studies long time ago by Ginsburg and co-workers (see [8–10] for some examples), although to our knowledge no such studies have been reported for the eukaryotic enzyme. In this paper, we show the use of isothermal titration calorimetry (ITC) to obtain the thermodynamic parameters for adenosine-5′-triphosphate (ATP) binding to wild-type *P. vulgaris* GS.

2. Experimental

2.1. Reagents

$BaCl_2$ (Aldrich, 99% pure) and 18-crown-6 (Aldrich, 99.5% pure) were kept in a vacuum desiccator with P_2O_5. Bovine liver glutamate dehydrogenase (GDH) was obtained from Sigma as a 32 mg ml^{-1} suspension in 2.0 M $(NH_4)_2SO_4$, pH 7.0 and was stored at 4 °C. ATP (Sigma, disodium salt, minimum 99%) and ADP (Sigma, sodium salt, 98%) were purchased as desiccated powder and stored at −20 °C. Freshly made solutions were utilised for each titration experiment. All other chemicals were analytical reagent grade.

2.2. Overexpression of plant glutamine synthetase in E. coli

The *Bgl*II–*Hin*dIII fragment of the plasmid pcGS-18α [5], corresponding to the coding region of wild-type *P. vulgaris* α GS, was PCR amplified and cloned into the plasmid pQE30 (Quiagen), generating N-terminal polyhistidine-tagged fusion proteins. Recombinant plasmid was then transformed into the SG13009 *E. coli* strain. Transformants were used to inoculate 2.5 l cultures as described in [11] and cells were harvested by centrifugation after appropriate culture time and stored at −80 °C.

2.3. Purification of recombinant protein

Pellets of *E. coli* cells were thawed in extraction buffer and bacteria were lysed with 10 sonication cycles of 20 s each. The crude extract so obtained was applied to a Ni–Nta agarose matrix (Quiagen), which selectively binds polyhistidine-tagged proteins [12]. Elution was conducted, after two washes with 10 and 20 mM imidazole, respectively, in 10 mM Tris–HCl, pH 7.50 (at 25 °C), 10 mM $MgCl_2$ and 200 mM imidazole (adjusted to pH 8.0). Eluted protein was concentrated using Millipore Ultrafree-4 columns. Purified GS was dialysed against 1000 volumes of 10 mM Tris–HCl, pH 7.50, containing 10 mM $MgCl_2$, with several buffer changes. Aliquots of GS were frozen at −80 °C until use. All the steps of the purification process were done at 4 °C in a cold room or in a refrigerated Beckman J2-21 centrifuge. Protein samples for ITC measurement in Hepes buffer were prepared using the same protocol but replacing Tris with 10 mM Hepes–HCl, pH 7.50 in purification and dialysis steps. Using this procedure, the protein was confirmed to be purified to electrophoretic homogeneity as described in [11].

2.4. Enzyme assays

GS catalyses, in vivo, the biosynthesis of L-glutamine, in the presence of magnesium or manganese (Me^{2+}), according to the following equation:

$$\text{L-glutamate} + \text{ATP} + NH_4{}^+ \xrightarrow{Me^{2+}} \text{L-glutamine} + \text{ADP} + \text{Pi} + H^+ \quad (1)$$

We refer to this reaction as the “biosynthetic activity”. The standard GS biosynthetic assay contained in a final volume of 100 ml:10 μmol Tris–HCl, pH 7.50 (at 25 °C); 10 μmol L-glutamate; 5 μmol NH_4Cl; 5 μmol $MgCl_2$; 0.75 μmol ATP (from a 100 mM stock at pH 7.50) and 5–10 ng of purified enzyme. The assay mix-

ture was incubated for 10 min at 37 °C and the inorganic phosphate released from ATP hydrolysis was determined using the green malaquite method [13]; blanks were identical but lacking L-glutamate.

GS is also commonly assayed through an arsenolysis side reaction named "transferase activity" [14]:

$$\text{L-glutamine} + NH_2OH \xrightarrow[Me^{2+}]{ADP,As} \gamma\text{-glutamylhydroxamate} + NH_4{}^{+} \quad (2)$$

Standard reaction mixture for GS transferase assay contained in a final volume of 1 ml: 120 μmol MOPS buffer, pH 7.0; 90 μmol L-glutamine; 2.4 μmol $MnCl_2$; 50 nmol ADP; 120 μmol hydroxylammonium chloride neutralised with 60 μmol NaOH; 50 μmol DTE; 50 μmol of Na_2HAsO_4 and 50–100 ng of purified enzyme. The reaction was incubated for 10 min at 37 °C after which the product γ-glutamylhydroxamate was revealed as described in [15]; blanks were identical but lacking arsenate.

K_m values for ATP were determined by using the standard biosynthetic assay but varying the concentration of ATP while keeping the other substrates at the same concentration. Linear regression analysis of double reciprocal data was performed using Sigma plot (SPSS Inc.)

2.5. Isothermal titration calorimetry

A multichannel thermal activity monitor (TAM) isothermal heat conduction microcalorimeter (Thermometric AB 2277/201, Järfälla, Sweden) was used, this instrument have been described in details by Suurkuusk and Wadsö [16]. The microcalorimeter was connected to an external water circulator (Heto) and the whole system was placed in a room in which the temperature was kept constant within ±0.5 °C. An 1.1 ml titration vessel was equipped with a stirring system. The vessel was loaded with 0.8 ml of protein solution using a Hamilton syringe, thermostated at 25 °C and continuously stirred at 60 rpm. Ligand was injected through a stainless steel cannula connected to a 250 μl Hamilton syringe, which was positioned in a computer controlled syringe pump (Hamilton Microlab M). Injections were made over a period of 10 s with intervals of 6 min. The experiment was computer controlled using Digitam 4.1 software (Thermometric); the same program was used for data analysis. The microcalorimeter was electrically calibrated before each titration experiment. Nevertheless, in order to check the performance of our system, the binding of Ba^{2+} (aq) to 18-crown-6 was used. The results obtained, $K_c = 5667 \pm 680\ M^{-1}$ and $\Delta H_m = -(31.46 \pm 0.70)\ kJ\ mol^{-1}$ are in agreement with those reported by Briggner and Wadsö [17]: $K_c = 5900 \pm 200\ M^{-1}$ and $\Delta H_m = -(31.42 \pm 0.20)\ kJ\ mol^{-1}$. Since the system under study belongs to a protein–ligand binding process, we used the binding of ADP to bovine liver GDH as a second system test. The enzyme was prepared as described in [18].

Samples of purified GS to be used for ITC were centrifuged to remove any undissolved material and briefly degassed before use; to minimise dilution artefacts ATP was dissolved in the same dialysis buffer of GS and the pH of ATP solution was adjusted to 7.50 (the same of protein solution) with NaOH. A separate experiment was run for each titration in order to determine the heat of dilution of ATP in GS dialysis buffer. Concentration of GS and ATP were determined by measuring respectively the absorbance at 280 nm (ε = 462,000 $M^{-1}\ cm^{-1}$ for native octamer) and 259 nm (ε = 15,400 $M^{-1}\ cm^{-1}$). The data reported are the mean of two measures made with different protein preparations; experiments were repeated more than twice if the obtained parameters were not congruent.

3. Results and discussion

3.1. Calorimeter test with GDH

Fig. 1 shows the results of a typical titration experiment of binding of ADP to GDH. The thermodynamic parameters obtained were compared with those reported in the literature [19] (Table 1). Considering the complexity of that system, the data are in good agreement to those proposed by Subramanian et al.,

Table 1
Main thermodynamic parameters estimated for ADP binding to GDH

	[19]	This work
ΔH ($kJ\ mol^{-1}$)	51.9 ± 0.84	50.2 ± 1.6
K (M^{-1})	(2.66 ± 0.07) × 10^5	(2.76 ± 0.11) × 10^5

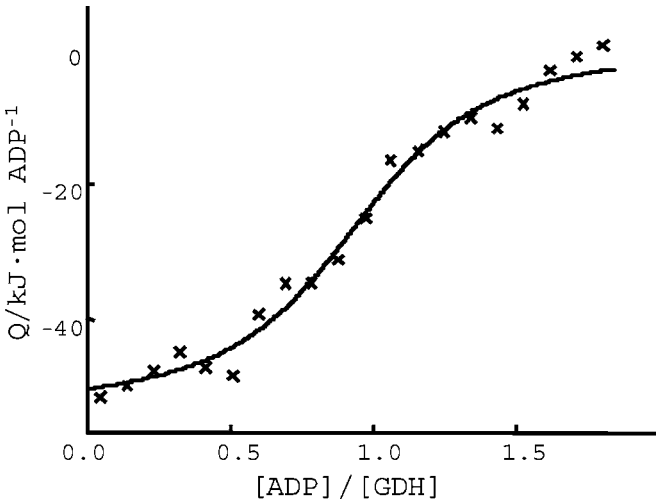

Fig. 1. Normalised integrated heats, corrected for dilution, for binding at 25 °C of ADP (6 mM, 20 injections of 5 μl) to 0.9 ml of bovine liver GDH (0.37 mM) in 100 mM phosphate buffer, pH 7.60. Data were plotted to a one binding site model.

confirming that this system could be used as an alternative "biochemical" way to check the instrument.

It has been shown that freezing the protein preparations lead to partial inactivation of GDH [20]. In our case, a very small response in terms of heat developed upon ADP binding was obtained when using freeze-thawed GDH. This result suggests that freeze-thawed inactive GDH enzyme is possibly unable to bind ADP. On the other hand, all results confirmed that the equipment was ready to accurately measure the binding process of other substrates and protein molecules.

3.2. Thermal stability of wild-type GS

In a typical isothermal calorimetric run, using our experimental system, the sample must stay stable at the calorimeter working temperature for about 4 h. GS enzymes have been reported as thermally unstable proteins [21]. For this reason it was a strict requirement of the present work, before planning ITC experiments, to study the stability of the wild-type purified homopolymeric GS in a range of possible working temperatures for microcalorimetry (from 4 to 37 °C). It was observed that the enzyme, at a concentration of 5 mg ml^{-1}, retains about 90% of initial activity after 24 h of incubation at 25 °C, while 50% of activity could be recovered during 1 h at 37 °C. Diluted preparations of enzyme were considerably more unstable. Therefore, 25 °C seemed a reasonable working condition for microcalorimetric studies of concentrated enzyme preparations. However, we have also considered the possibility of addition of ethylene glycol to further improve GS stability. Fig. 2 shows the effect of ethylene glycol and protein concentration on transferase and biosynthetic enzyme activities of GS upon incubation at 25 °C. It can be observed a clear stabilising effect of ethylene glycol which was proportional to its concentration, and that the dilution of GS (in the same enzyme buffer) affected negatively to the thermal stability. Transferase and biosynthetic enzyme activities of diluted GS were virtually zero after 50 h of incubation in the absence of ethylene glycol but the addition of this compound protected the enzyme against thermal inactivation. However, the concentrated enzyme transferase and biosynthetic activities were at least 90% retained after 24 h incubation (producing a gradual decrease in enzyme activity at longer incubations). Inactivation of GS in diluted preparations of the enzyme was also described by Denman and Wedler [22] for mammalian brain GS; the authors attributed the loss of activity to dissociation of octamer to tetramer and thence to inactive monomer in the absence of glycerol, added as stabilising compound. We found that glycerol could be used instead of ethylene glycol also in the case of plant GS,

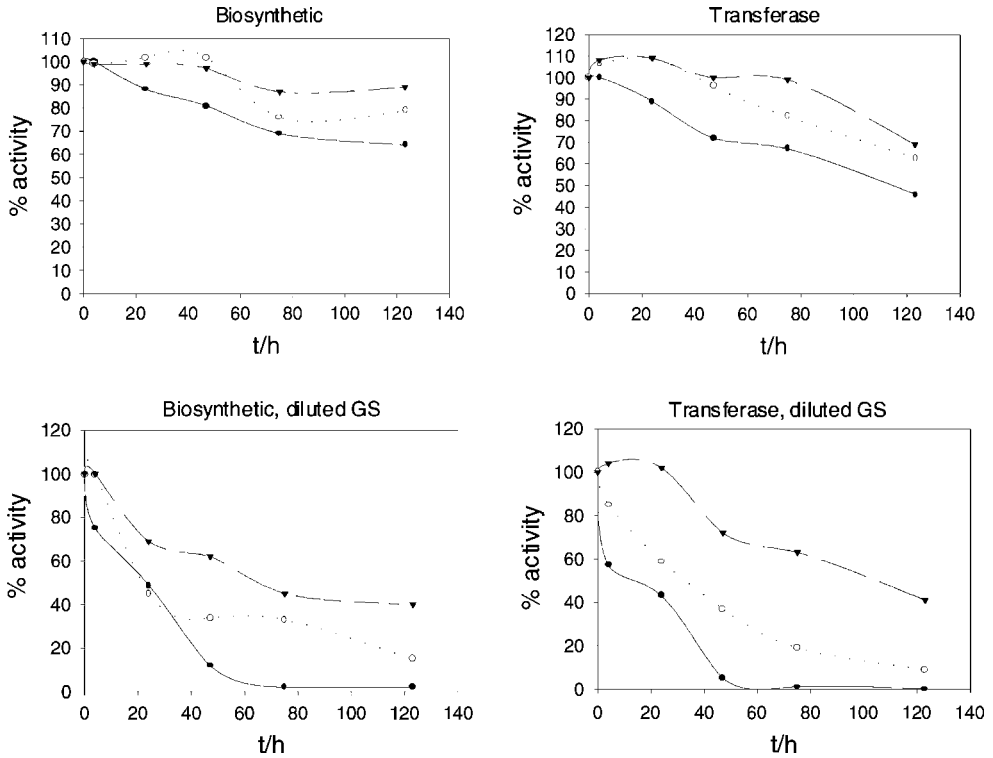

Fig. 2. Thermal stability of GS. Concentrated (5 mg ml^{-1}) and diluted (25 μg ml^{-1}) protein solutions were incubated at 25 °C in 10 mM Tris–HCl, pH 7.50, containing 10 mM $MgCl_2$ in the presence of different concentrations of ethylene glycol: (▼) 25% (v/v); (○) 10% (v/v); (●) 0% (v/v). Samples for determination of biosynthetic and transferase activity were taken at the indicated times and assays were carried out as explained in experimental.

producing a similar stabilising effect. However, ethylene glycol was preferred in this work due to its lower viscosity. Other authors commonly use ethylene glycol or glycerol as protecting agents in purified preparations of GS from their natural biological sources [23].

Finally, we also found that GS samples did not lose any significant activity after long time storage at −80 °C. Repeated cycles of freeze thawing did neither affect to enzyme activity. All this information was of crucial importance for handling and storing GS protein samples in the appropriate way, in order to start different sets of microcalorimetric measurements.

3.3. ATP binding to GS

The first titrations of GS with ATP were conducted at 25 °C using a 10 mM Tris–HCl pH 7.50 buffer containing 10 mM $MgCl_2$ and 25% (v/v) ethylene glycol, conditions previously found for maximal thermal stability of the enzyme. Heat values obtained from such experiments were too small (very close to the dilution of ATP) precluding any correct determination of K or ΔH. To test any possible interference of ethylene glycol with substrate binding, we made assays of biosynthetic GS activity in the usual reaction mixture (see Section 2) but containing the proportions of this stabilising compound present in the enzyme storage buffer and therefore, in the binding reaction mixture. Table 2 shows that the enzyme activity of the sample in the presence of 25% ethylene glycol was only 22% of the control. Lower amounts of ethylene glycol produced also lower but noticeable inhibition of enzyme activity. Results obtained are not contradictory to those shown in Fig. 2 when using ethylene glycol as stabilising compound, since in that case assays of GS activity were conducted by adding only a very small volume

Table 2
Effect of ethylene glycol on the biosynthetic activity of GS

Ethylene glycol concentration (% (v/v))	Biosynthetic activity
0	100
10	66
25	22

of protein solution containing ethylene glycol (1–5 μl) to the reaction mix. Thus, the quantity of ethylene glycol in the enzyme reaction was practically negligible. Therefore, we can say that, although ethylene glycol improves the stability of GS, it is also a potent inhibitor of it, being, therefore, inadequate for substrate binding experiments. Consequently, binding experiments had to be conducted in buffer lacking ethylene glycol, at 25 °C and using concentrated enzyme.

Fig. 3 shows the calorimetric response for titration of GS with ATP, the dilution of ATP (left) and the fitted data (right) for an experiment conducted in buffer 10 mM Tris–HCl, pH 7.50, containing 10 mM $MgCl_2$. Enzyme activity assays were carried out before and after titrations confirming that 95% of activity was maintained.

Any change in the protonation state of the protein upon binding of ATP will influence the observed binding enthalpy (ΔH_{obs}) in a way that is proportional to the heat of ionisation of the buffer used (ΔH_{ion}). We can formalise this with the following equation, also described by [24,25]:

$$\Delta H_{obs} = \Delta H_0 + \delta H^+ (\Delta H_{ion}) \quad (3)$$

where δH^+ is the number of protons that are released (if $\delta H^+ > 0$) by the buffer and ΔH_0 is the binding enthalpy in the absence of buffer effects. To quantify an eventual contribution of buffer ionisation to binding enthalpy we performed titrations both in Tris and Hepes buffers, at the same concentration and pH, which have ionisation enthalpies at 25 °C of 46.44 kJ mol^{-1} [26] and 21.01 kJ mol^{-1} [27], respectively. Using this approach we determined an uptake of 0.86 ± 0.08 protons from GS upon binding of ATP.

The effect of ionic strength on the binding process of ATP to homopolymeric GS was also studied by adding 100 mM KCl to the GS buffer. Titrations were again performed both in Tris and Hepes buffer. Proton uptake was, in this case, 0.58 ± 0.04. Changes in proton uptake from GS in different buffers could be attributed to changes in the pK_a values of amino acid residues due to the fact that ionic strength influences the activity coefficients. Data obtained from ITC measurements are summarised in Table 3. The binding process is clearly endothermic and so entropy-driven. The addition of 100 mM KCl, besides to change δH^+, also lowers the value of ΔH_0. Entropy-driven binding of ATP was also observed, for the *E. coli* (type I) enzyme, by Ginsburg et al. [8], which determinate a Van't Hoff enthalpy value of 12.6 ± 8.4 kJ mol^{-1}.

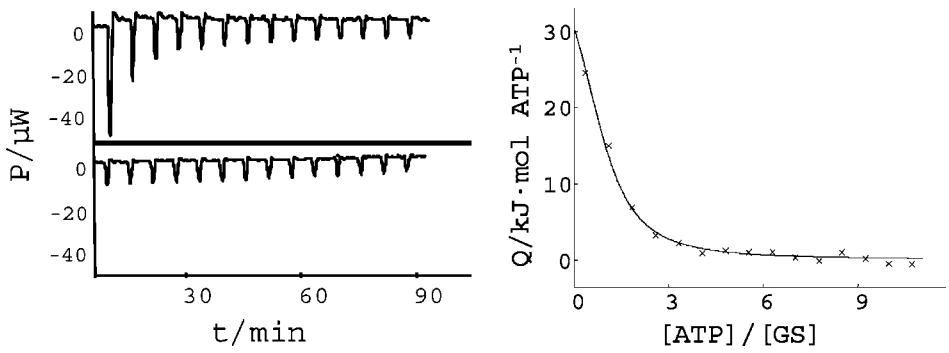

Fig. 3. On the left: titration of GS (6 mg ml^{-1}) by 10 mM ATP at 25 °C (upper graph) and dilution of 10 mM ATP (lower graph). Protein and ligand were in 10 mM Tris–HCl, pH 7.50, containing 10 mM $MgCl_2$; the pH of ATP solution was pre-adjusted to 7.50 with NaOH. Both titrations consisted in 14 injections of 10 μl. The peaks represent the inverse of the heat change of the system; in this case both binding and dilution were endothermic. The area under each peak was integrated and corrected for the corresponding dilution heat, the resulting data were changed of sign and plotted against the molar ratio of ATP to GS monomer as shown in the right part of the figure.

Table 3
Summary of thermodynamical parameters associated to binding of ATP to GS

	Tris–HCl	Hepes–HCl	Tris–HCl (with KCl 100 mM)	Hepes–HCl (with KCl 100 mM)
ΔH_{obs} (kJ mol^{-1})	44.8 ± 1.5	22.8 ± 3.3	30.8 ± 0.4	16.0 ± 2.3
K_{app} (M^{-1})	12890 ± 1350	4800 ± 880	2450 ± 420	17800 ± 4500
δH^+	0.86 ± 0.08		0.58 ± 0.05	
ΔH_0 (kJ mol^{-1})	4.80 ± 0.43		3.77 ± 0.30	

The calorimetric data obtained for plant GS fitted better to an n value of 1 ± 0.1 than 0.5 (n is the number of molecules of ligand that bind to each subunit of protein). This result is important since some researchers have found that each subunit of the octameric GS molecule can bind a molecule of ligand [28,29] while others proposed a "half-site reactivity" model for eukaryotic GS in which only four active centres are present in the octameric molecule [30,31].

Changes in values of the binding constants shown in Table 3 can be attributed to the particular experimental conditions in which binding experiments were conducted. The c value, which is obtained by multiplying the binding constant by the macromolecule concentration, was in our case of 1–1.5 (considering a K order of magnitude value of 10^4). Unfortunately, in these conditions a relevant error is expected from the computerised fitting of data to a binding model [32,33]. In our work we have to say that the absolute values of K_{app} are probably accurate within a factor of three. Other errors in the determinations of thermodynamic parameters could be attributed to the quantification of GS concentration since inactive molecules eventually present in the titration process (in spite of prior centrifugation) would contribute to the absorbance at 280 nm but not to ATP binding. Ligand concentrations were confirmed by A_{259} measurement and thought to be accurate ±2%.

Results obtained from calorimetric measurements were also contrasted with kinetic estimates of K_m for

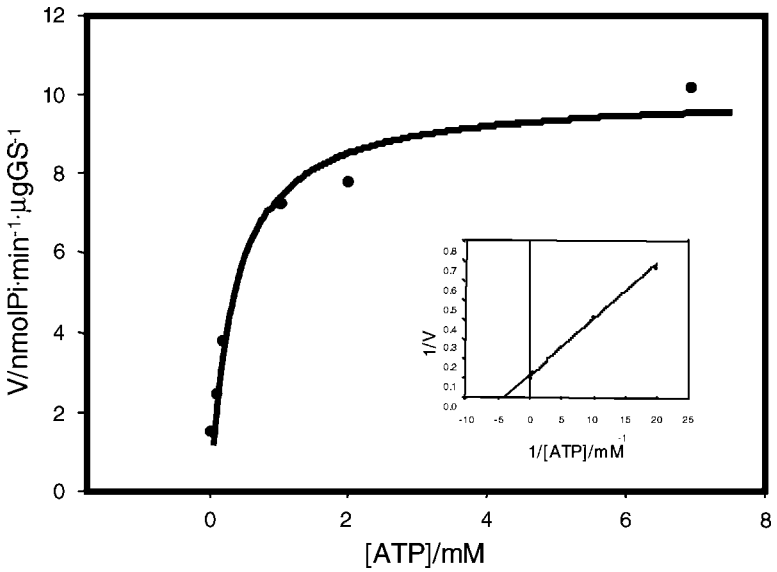

Fig. 4. Michaelis–Menten plot of reaction velocity vs. [ATP] and double reciprocal plot (inner graph) for the Mg^{2+} supported biosynthetic reaction at 37 °C. Assay conditions were as described in experimental.

Table 4
Some kinetic parameters for ATP-driven biosynthetic and transferase reactions of homopolymeric recombinant GS

Parameter	Value
Biosynthetic activity	
Specific activity ($U\,mg^{-1}$)	9.1 ± 0.7
K_m for ATP (mM)	0.25 ± 0.03
V_{max} (nmol Pi $min^{-1}\,\mu g^{-1}$ GS)	8.93 ± 0.65
k_{cat} or turnover number (s^{-1})	51.32 ± 3.75
Turnover number per active site (s^{-1})	6.42 ± 0.47
Time for one catalytic cycle per subunit (s)	0.16 ± 0.01
Catalytic efficiency ($mM^{-1}\,s^{-1}$)	205.28 ± 39.62
Transferase activity	
Specific activity ($U\,mg^{-1}$)	381 ± 23
V_{max} (nmol γ-glutamylhydroxamate $min^{-1}\,\mu g^{-1}$ GS)	547 ± 33
k_{cat} or turnover number (s^{-1})	3.136 ± 188
Turnover number per active site (s^{-1})	392 ± 24
Time for one catalytic cycle per subunit (s)	$(2.6 \pm 0.2) \times 10^{-3}$
Transferase/biosynthetic enzyme activity ratio	41.9

ATP. Although K_m values for ATP have been repeatedly determined for purified preparations of GS from different natural sources, it was never tested in the homopolymeric recombinant form used in this work. Fig. 4 shows velocity–ATP concentration and double reciprocal plots which yield a calculated estimate of K_m for ATP corresponding to 0.25 ± 0.03 mM, this value is in the same order of magnitude than other natural and recombinant forms of GS from plants [1–7]. Considering that K_m reflects the inverse of the affinity of the enzyme towards its substrate, the result obtained is in good agreement with the order of magnitude of previous estimates made by ITC of the ATP binding constants. A k_{cat} value of $51.23 \pm 3.75\,s^{-1}$ can be also deduced from the V_{max} estimate from the double reciprocal plot of Fig. 4. This means that an enzyme cycle can be completed within 0.16 ± 0.01 s. Table 4 summarises some kinetic parameters for both biosynthetic and transferase activities of homopolymeric recombinant GS.

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