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Structure and Mechanism of an ADP-Glucose Phosphorylase from *Arabidopsis thaliana*,^{†,‡}

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Abstract

The X-ray crystal structure of the At5g18200.1 protein has been solved to a nominal resolution of 2.30 Å. The structure has a histidine triad (HIT)-like fold containing two distinct HIT-like motifs. The sequence of At5g18200.1 indicates a distant family relationship to the *Escherichia coli* galactose-1-P uridylyltransferase (GalT): the determined structure of the At5g18200.1 protein confirms this relationship. The At5g18200.1 protein does not demonstrate GalT activity but instead catalyzes adenylyltransfer in the reaction of ADP-glucose with various phosphates. The best acceptor among those evaluated is phosphate itself, thus the At5g18200.1 enzyme appears to be an ADP-glucose phosphorylase. The enzyme catalyzes the exchange of ¹⁴C between ADP-[¹⁴C]glucose and glucose-1-P in the absence of phosphate. The steady state kinetics of exchange follows the ping pong bi bi kinetic mechanism, with $k_{cat} = 4.1 \text{ s}^{-1}$ and K_m -values of 1.4 μM and 83 μM for ADP-[¹⁴C]glucose and glucose-1-P, respectively, at pH 8.5 and 25 °C. The overall reaction of ADP-glucose with phosphate to produce ADP and glucose-1-P follows ping pong bi bi steady state kinetics, with $k_{cat} = 2.7 \text{ s}^{-1}$ and K_m -values of 6.9 μM and 90 μM for ADP-glucose and phosphate, respectively, at pH 8.5 and 25 °C. The kinetics are consistent with a double displacement mechanism that involves a covalent adenylyl-enzyme intermediate. The X-ray crystal structure of this intermediate was solved to 1.83 Å resolution, and shows the AMP-group bonded to His¹⁸⁶. The value of K_{eq} in the direction of ADP and glucose-1-P formation is 5.0 at pH 7.0 and 25 °C in the absence of a divalent metal ion, and it is 40 in the presence of 1 mM MgCl₂.

The At5g18200.1 sequence belongs to the histidine triad (HIT) superfamily of nucleotide hydrolases and transferases (Superfamily E = 3.0e-45 and E = 1.0e-29 for the C and N terminal portions, respectively) (1). This superfamily contains three distinct families: the histidine triad nucleotide-binding protein family, the fragile histidine triad family, and the GalT family (2).

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[‡]X-ray coordinates for the Arabidopsis At5g18200.1 protein have been deposited in the Protein Data Bank under PDB codes 1ZWJ and 1Z84

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¹The abbreviations are: ADP, adenosine-5'-diphosphate; ADP-glucose, adenosine-5'-diphosphate-α-D-glucose; UDP-glucose, uridine-5'-diphosphate-α-D-glucose; UDP-galactose, uridine-5'-diphosphate-α-D-galactose; NAD⁺, nicotinamide adenine dinucleotide; NADP⁺, nicotinamide adenine dinucleotide phosphate; NADH, reduced NAD⁺; NADPH, reduced NADP⁺; glucose-1-P, α-D-glucose-1-phosphate; galactose-1-P, α-D-galactose-1-phosphate; mannose-1-P, α-D-mannose-1-phosphate; GDP-mannose, guanosine-5'-diphosphate-α-D-mannose; HEPES, N-(2-hydroxyethyl)piperazine-N'-(4-butanedisulfonic acid); EDTA, ethylenediamine tetraacetic acid; GalT, galactose-1-phosphate uridylyltransferase; HIT, histidine triad; P_i, inorganic phosphate; PEG, polyethylene glycol; SAD, single wavelength anomalous dispersion; MES, 2-(n-morpholino)ethanesulfonic acid;

The At5g18200.1 enzyme is a member of the GalT family, which differs from the other two families in several respects: it does not contain a “triad of histidines” but rather a conserved HXHXQ sequence, and the typical reaction catalyzed by enzymes of this family is nucleotidyl transfer rather than hydrolysis (3). The GalT family contains three known subfamilies of enzymes (3): the GALT-like UDP-hexose:hexose-1-P uridylyltransferases, an adenylylsulfate:phosphate adenylyltransferase from *Thiobacillus denitrificans* which converts adenosine-5'-phosphosulfate and phosphate to ADP and sulfate (4), and diadenosine tetraphosphate phosphorylase whose catalytic activity results in the formation of ATP and ADP (5).

In the genome of *Arabidopsis thaliana*, the locus At5g18200.1 (6) encodes a protein that shares amino acid sequence homology with GalT from *Escherichia coli* (PSI-BLAST E = 6e-21) (7). Due to its homology with GalT, the At5g18200.1 enzyme is annotated as a GalT-like protein. In particular, the motif His¹⁸⁴Ser¹⁸⁵His¹⁸⁶Ser¹⁸⁷Gln¹⁸⁸ in the At5g18200.1 protein is similar to the active site motif His¹⁶⁴Pro¹⁶⁵His¹⁶⁶Gly¹⁶⁷Gln¹⁶⁸ of GalT. The reaction catalyzed by GalT proceeds by a double displacement mechanism with a covalent uridylyl-enzyme intermediate, which involves the nucleophilic catalyst His¹⁶⁶ (8–10).

In this paper, we describe the X-ray crystal structure of the At5g18200.1 protein and a covalent adenylyl-enzyme intermediate. We further describe the catalytic properties of the protein and show that it is not a GalT, but an adenylyltransferase that uses ADP-glucose as the donor substrate and catalyzes adenylyltransfer to a broad range of phosphate acceptors. The best acceptor examined is phosphate itself, so the enzyme is an ADP-glucose phosphorylase. We demonstrate that the catalytic mechanism is analogous to that of GalT. We also show that the reaction preferably proceeds in the direction of the formation of ADP and glucose-1-phosphate from ADP-glucose and phosphate.

EXPERIMENTAL PROCEDURES

Materials

ADP-D-[U-¹⁴C]glucose was purchased from Amersham Biosciences. Activated charcoal, MgCl₂, bicine, and EDTA were obtained from Aldrich Chemical Company. ADP, phosphoenolpyruvate, NADH, pyruvate kinase, HEPES, and lactic dehydrogenase were purchased from Sigma. Potassium phosphate monobasic, KCl, NaCl, NaOH, and HCl were obtained from Fisher Scientific. Microcon centrifugal filter devices were purchased from Amicon/Millipore.

Protein Expression and Purification

The At5g18200.1 gene was cloned into a pVP13 plasmid encoding a histidine₆-maltose binding protein tag according to the *E. coli* based protocols described previously (11). Both unlabeled and selenomethionyl labeled proteins were expressed according to standard Center for Eukaryotic Structural Genomics protocol (12). Following sonication, the protein was purified via immobilized nickel affinity chromatography, and TEV protease was used to cleave the affinity/solubility tag (13). After tag capture and a final desalting step the selenomethionyl labeled protein was concentrated to 8.4 mg/mL and dialyzed against 50 mM NaCl and 5 mM HEPES pH 7.0. The unlabeled protein sample was concentrated to 10.0 mg/mL and dialyzed against 50 mM NaCl and 5 mM Tris pH 8.0.

Crystallization

Unlabelled and selenomethionyl At5g18200.1 protein crystals were grown by the hanging-drop vapor-diffusion method. The reservoir solution contained 20 % (w/v) PEG 2000, 200 mM NaCl, and 100 mM MES-acetate pH 5.5. Hanging drops consisted of 2 µL of protein solution

mixed with 2 μ L of reservoir solution. The crystals were grown at room temperature, and diffraction-quality specimens were obtained within two to three days. Crystals of the unliganded protein were then soaked in a series of reservoir solutions containing increasing amounts of ethylene glycol, up to a final concentration of 20 % ethylene glycol, and were then flash-frozen in a stream of nitrogen. To prepare derivative crystals with the ligand we first soaked them in a solution containing 20 % (w/v) PEG 2000, 200 mM NaCl, 2 mM ADP-glucose, and 100mM HEPES pH 7.5 for a period of two hours. Crystals were then cryoprotected and frozen as described above.

Data Collection and Reduction

Diffraction data for the native, unliganded structure were collected on beamline 14-ID-B BIOCARs at Argonne National Laboratories at a wavelength of 0.9786 Å. Two data sets for the selenomethionyl labeled, unliganded structure were collected on beamline 32-ID-B SBC at a wavelength of 0.9791 Å. The diffraction images were integrated and scaled using the HKL2000 package (14). Initial attempts to define the selenium substructure of the crystals failed. Therefore, a molecular replacement in Molrep (6,15) was attempted using the GALT dimer (PDB code 1gup) as a search model. A very weak solution was obtained that provided the initial phase information necessary for the location of the strongest anomalous scatterers. The selenium substructure was completed with several cycles of SAD-phasing and inspection of anomalous and gradient anomalous difference maps. The final set of anomalous scatterers was used for SAD-phasing in CNS (16). Initial phase information to 3.5 Å was further improved and extended to the 2.4 Å resolution of the native data set by density modification and phase extension as implemented in CNS. The structure was built from the GALT dimer model and completed using alternate cycles of manual building in O (17) and Xfit (18) and crystallographic refinement in CNS. The native, liganded structure data were collected on beamline 19-BM SBC-CAT at Argonne National Laboratories at a wavelength of 0.97948 Å. HKL2000 was again used to integrate and scale the diffraction data, and initial phases were determined with MOLREP using the unliganded At5g18200.1 structure as the search model. Refinement was carried out using REFMAC (15,19) and the model was built with Xfit.

Assays

Adenylyltransferase activity was initially measured by the coupled assay employed for GalT, substituting ADP-glucose for UDP-glucose and using galactose-1-P, mannose-1-P, or P_i as the acceptor substrate (8). UV-visible spectrophotometry was performed on a Hewlett-Packard model 8452A diode array spectrophotometer.

The rate of glucose-1-P formation was measured by a coupled assay using $NADP^+$, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The assay was carried out in 1.0 mL containing 50 mM HEPES pH 8.0, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM $NADP^+$, 0.5 μ M glucose-1,6-diphosphate, 0.1 U/ml phosphoglucomutase, and 0.1 U/ml glucose-6-phosphate dehydrogenase. The increase in A_{340} due to NADPH-formation was used to calculate the glucose-1-P content of the aliquot, using $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ as the extinction coefficient.

The rate of ADP formation was measured enzymatically. The assay was carried out in 1.0 mL containing 50 mM HEPES pH 7.0, 2 mM $MgCl_2$, 75 mM KCl, 0.2 mM NADH, 0.3 mM phosphoenolpyruvate, 1 U/ml lactate dehydrogenase, and 1 U/ml pyruvate kinase. The decrease in A_{340} due to consumption of NADH was used to calculate the amount of ADP in the aliquot, with $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ as the extinction coefficient.

Methods

The extinction coefficient for the At5g18200.1 protein at 280 nm was determined by the method of Gill and von Hippel (20). First, the value of ϵ_{280} for the denatured protein in a 6 M guanidine-HCl (ϵ_{den}) was calculated from the number of tryptophan, tyrosine, and cysteine residues per molecule of the protein and their respective values of ϵ_{280} . Then absorbances at 280 nm of the native (Abs_{nat}) and denatured (Abs_{den}) proteins were measured at identical protein concentrations. The extinction coefficient for the native protein (ϵ_{nat}) was calculated using the equation: $\epsilon_{\text{nat}} = (\text{Abs}_{\text{nat}})(\epsilon_{\text{den}}) / (\text{Abs}_{\text{den}})$. The value determined for the At5g18200.1 monomer ϵ_{nat} was $32.0 \pm 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$.

The equilibrium constant for the reaction of ADP-glucose with P_i to produce ADP and glucose-1-P was evaluated as follows. The reaction was allowed to proceed for 20 min to reach equilibrium followed by heating in boiling water for 5 min to inactivate the enzyme. The reaction mixtures (2.0 mL) initially contained 0.1 M HEPES pH 7.0, 0.1 mM P_i , 0.05 mM ADP-glucose, and $25 \mu\text{g mL}^{-1}$ of ADP-glucose phosphorylase. After centrifugation of the coagulated enzyme (10 min at 14,000 rpm) aliquots of the supernatant fluid were enzymatically assayed for glucose-1-P and ADP content. To study the effect of Mg^{++} , the experiment was also performed in the presence of 1 mM MgCl_2 . These experiments were carried out under variable concentrations of substrates and enzyme and also for longer reaction times. The results showed that equilibrium had been attained under all conditions employed.

Steady state kinetics

A radiochemical method for assaying GalT (8) was adapted to measuring the initial rates of the reaction of ADP-glucose with P_i to form ADP and glucose-1-P. Each reaction mixture (1.5 mL) contained 0.1 M Na-bicinate at pH 8.5, 5–60 μM inorganic phosphate, 0.2–7.0 μM ADP- ^{14}C glucose (3.7×10^6 – $2.6 \times 10^7 \text{ cpm } \mu\text{mol}^{-1}$) and 6.7 nM At5g18200.1 enzyme. The reactions were started by addition of the enzyme. At pre-determined times, aliquots of the reaction mixture were added to 20 mg of activated charcoal and mixed thoroughly to stop the reaction. The mixtures were centrifuged and the supernatant fluids filtered, using Microcon YM-50 centrifugal filter devices. Aliquots of filtrates were assayed radiochemically for ^{14}C using liquid scintillation counting on a Beckman LS 6500 spectrometer. Progress curves were linear and gave the initial rates.

The initial rates for the exchange of ^{14}C glucose between ADP-glucose and glucose-1-P were measured by the charcoal-quench method described above for the overall reaction. In this case the reaction mixtures (1.5 mL) contained 0.1 M Na-bicinate at pH 8.5, 10–400 μM glucose-1-P, 0.25–1.0 μM ADP- ^{14}C glucose ($2.8 \times 10^7 \text{ cpm } \mu\text{mol}^{-1}$) and 8.0 nM At5g18200.1 enzyme. The linear progress curves yielded initial rates.

Data Analysis

The initial rate data were computer fitted to eq. 1 for the ping pong mechanism using PNGPNG program of Cleland (21):

$$v = V_m [A][B] / \{K_a[B] + K_b[B] + [A][B]\} \quad (1)$$

In this equation A and B refer to ADP-glucose and P_i , respectively; values of v are the experimental initial rates. k_{cat} values were obtained through the division of V_m -values by the enzyme concentration.

RESULTS

Structure Determination

The structure of the At5g18200.1 protein has been solved to a resolution of 2.30 Å. The relevant statistics are given in Table 1. The protein forms a dimer with each chain comprising 351 amino acids. The asymmetric unit contains the biological homodimer, labeled as chains A and B. Several surface loops were not modeled due to insufficient electron density. These include amino acids 1–22, 40–47, 52–61, and 106–115 of chain A, as well as residues 1–20, 40–45, 51–61, 68–71, 106–115, and 351 of chain B. A Ramachandran plot indicates 557 of the 600 modeled amino acids are in the most favored phi/psi regions with the remainder falling in the generously allowed regions. The asymmetric unit also contains 225 ordered water molecules. Each monomer in the asymmetric unit coordinates two zinc atoms via two cysteine and two histidine sidechains.

Protein Fold

The overall fold of the monomer is shown in Figure 1 and consists of an antiparallel open faced β -pleated sheet formed by β -strands C, D, F, H, G, L, M, K, J, and I which surround two antiparallel amphipathic helices (5 and 10) which overlap near their N terminals forming an angle of roughly 50°. Short helices (4 and 9) are positioned at roughly 90° from the primary axes of helices 5 and 11 such that their amino terminals point directly towards the β -sheet. The remaining β -strands form a mixed intersubunit β -sheet which consists of strands A, B, and N of one monomer and strand E from the other. Strand B also makes contacts with a type I turn from the opposing chain. In addition, the protrusion containing helix 13 makes extensive contacts with alpha helix 6, the loop running from the carboxy terminal of strand H to the amino terminal of helix 7, and β strands M, L, and G of the other monomer (see Figure 2). The average buried solvent accessible surface area of the interface is 1955 Å².

Reaction catalyzed

Since the At5g18200.1 protein is annotated as a GalT-like nucleotidyltransferase, the enzyme was tested for GalT activity. GalT catalyzes the conversion of UDP-glucose and galactose-1-P into glucose-1-P and UDP-galactose. The production of glucose-1-P forms the basis for the standard assay (8). The coupled assay includes phosphoglucomutase, glucose-6-P dehydrogenase and NADP⁺ and measures glucose-1-P formation through formation of NADPH. Surprisingly, the At5g18200.1 protein displays less than 5×10^{-5} the activity of *E. coli* GalT. Therefore we performed detailed substrate specificity studies. These studies reveal adenylyltransferase activity with ADP-glucose as the donor and galactose-1-P as the acceptor. Typical results shown in Table 2 indicate that the At5g18200.1 enzyme is almost ten-times more active in the GalT assay with ADP-glucose as the donor substrate, and it is another 2.8 times more active with mannose-1-P as the acceptor substrate. The preferential utilization of ADP-glucose and mannose-1-P rather than UDP-glucose and glucose-1-P clearly shows that the At5g18200.1 enzyme does not possess GalT activity. ADP-glucose and inorganic phosphate are the best donor/acceptor pair of substrates so far tested. The entries in Table 2 show the activity with ADP-glucose and P_i to be more than 700 times that with UDP-glucose and galactose-1-P. The enzyme does not display ADP-glucose pyrophosphorylase activity, as shown by the fact that MgPP_i does not serve as an acceptor.

In preliminary experiments to narrow the focus on substrate preference, apparent values of $V_{\max}$ and K_m confirm and quantify the preferences implied by the survey data in Table 2. Assaying for glucose-1-P production from ADP-glucose, at 1 mM galactose-1-P, the value of K_m^{app} for ADP-glucose is 7 μM , and at 5 μM ADP-glucose the value of K_m^{app} for galactose-1-P is 2 mM. In contrast, the value of K_m^{app} for P_i is 18 μM at 20 μM ADP-glucose. Comparing rates, at 5 μM ADP-glucose, the value of the apparent second-order rate constant (k_{cat} /

K_m^{app} for the reaction of galactose-1-P is $3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, and that for the reaction of P_i at $20 \mu\text{M}$ ADP-glucose is $4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. These constants cannot be compared directly because of the four-fold difference in concentrations of ADP-glucose. However, even if the value for P_i were four-fold less—this would be the maximum normalization—the ratio of apparent second-order rate constants would be more than 300 in favor of the reaction of P_i . It must be concluded that the At5g18200.1 enzyme is not a GalT, nor is it an ADP-glucose pyrophosphorylase, but it is an adenyltransferase that displays selectivity for ADP-glucose and P_i as donor and acceptor substrates.

Kinetic mechanism

The reactions catalyzed by the At5g18200.1 enzyme and GalT are chemically similar, in that both catalyze nucleotidyl transfer with nucleotide sugars as donor substrates and phosphates as acceptors. Therefore, given the sequence and structure homology, the chemical and kinetic mechanisms might be analogous. If so, the adenyltransferase should function by a double displacement chemical mechanism and ping pong kinetics through a covalent nucleotidyl-enzyme intermediate, as does GalT. Alternatively, the reaction might proceed by a sequential kinetic mechanism characteristic of UDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase, with no covalent intermediate. Steady state kinetic analysis distinguishes these mechanisms.

Equations 2 and 3 describe the hypothetical double displacement mechanism with ping pong kinetics for the action of the At5g18200.1 enzyme, where His186 is the central histidine in the putative catalytic motif His¹⁸⁴Ser¹⁸⁵His¹⁸⁶Ser¹⁸⁷Gln¹⁸⁸.

According to this mechanism, the enzyme must catalyze the exchange of ^{14}C between ADP-[^{14}C]glucose and glucose-1-P in the absence of P_i with ping pong kinetics and at a rate that is compatible with the rate of the overall reaction. The At5g18200.1 enzyme satisfies both criteria, as shown in Figure 3, where the reciprocals of the initial rates of exchange are plotted against the reciprocal of the concentration of glucose-1-P at three fixed concentrations of ADP-[^{14}C]glucose. The observation of exchange supports eq. 2 as a step in the kinetic mechanism, and the parallel line pattern supports the ping pong kinetic mechanism with a covalent AMP-enzyme as an intermediate. The kinetic parameters for the exchange reaction, obtained by fitting the data presented in Figure 3 to eq. 1 for the ping pong kinetic mechanism, are given in Table 3.

If the kinetic and chemical mechanism of eqs. 2 and 3 is correct, the steady state kinetics of the overall reaction must also conform to eq. 1. As shown in Fig. 4, the double reciprocal plot of steady state kinetic data is a set of parallel lines corresponding to eq. 1. When fitted to the data in Fig. 4, the kinetic parameters in Table 3 are obtained. Comparison with the parameters for the exchange ADP-[^{14}C]glucose/glucose-1-P show that the exchange reaction is the faster, so that the exchange rate is kinetically compatible with the rate and mechanism of the overall reaction. The kinetics of the exchange and overall reactions strongly support the ping pong kinetic mechanism and the chemistry of eqs. 2 and 3, and they rule out all sequential kinetic mechanisms.

According to the kinetic mechanism, the reaction of the At5g18200.1 enzyme with ADPglucose must produce the covalent adenyl-enzyme intermediate. In the present study, this intermediate is isolated and characterized by X-ray crystallography in work that is described in a later section.

Equilibrium constant (K_{eq})

The reaction of ADP-glucose with P_i to form ADP and glucose-1-P should be energetically reasonably balanced. We measured the value of K_{eq} at pH 7.0 in the absence of Mg^{2+} and found it to be 5.0 ± 0.3 . While the reaction to form glucose-1-P is fairly well favored, Mg^{2+} should draw it much further to completion because of the formation of MgADP and the relatively weak binding of Mg^{2+} to ADP-glucose. Accordingly, the value of K_{eq} in the presence of 1 mM $MgCl_2$ is 40 ± 1 . Thus, in the presence of 1 mM Mg^{2+} , the reaction is strongly favored in the forward direction, with the value of $\Delta G^{\circ} = -2.2 \text{ kcal mol}^{-1}$.

Crystallographic isolation of AMP-enzyme intermediate

The covalently modified enzyme structure was solved to a resolution of 1.83 Å (Table 1). The electron density for AMP was clearly evident in the electron density map. The α -phosphorus of AMP was refined to a position 1.8 Å from the NE2 of His186 in chain A and 1.7 Å in chain B. The coordination of AMP is identical in both chains A and B. A number of hydrogen bonds are observed coordinating the AMP molecule (see Figure 5). The sidechain amines of Gln188 and Asn173 both coordinate with one of the oxygens of the α -phosphate of AMP. In addition, a solvent molecule is within hydrogen bonding distance of both the oxygen and the backbone amine of Asn173 (not shown). Gln188 appears to make a second contact with the bridging oxygen of AMP as well. The other phosphate oxygen is within hydrogen bonding distance of the backbone amines of Ser181 and Met182. The sidechain of Asn96 is within hydrogen bonding distance of oxygens O2' and O3' of the ribose sugar. The adenine group is coordinated to the backbone amines of Ala74 and Leu95 and the backbone carboxyl group of Glu72. Additional stabilization is provided by "T stacking interactions" through the sidechains of Phe65 and Tyr94.

DISCUSSION

Protein Fold

The overall fold of the At5g18200.1 protein is quite similar to that of the *E. coli* GalT (DALI score = 31.8). The largest discernable difference between the two monomers is between residues 70 through 90 of the Arabidopsis protein and the analogous region in GalT. This region corresponds to strand C in the At5g18200.1 enzyme but in GalT forms an extended loop. A further difference is that the buried surface area between the At5g18200.1 monomers is only slightly more than half the value reported for the GalT dimer. The difference appears to be due mainly to the missing loops of the At5g18200.1 protein, which in GalT are involved in the dimer interface, but for which there was insufficient electron density to build into the At5g18200.1 protein model.

Zinc Binding Sites

In addition, there is a difference in metal binding between the two enzymes. The *E. coli* GalT monomer contains both a zinc and an iron atom (22); the identities of these metals have been confirmed by inductively coupled plasma emission spectroscopy (23). It has been further shown that enzyme activity is dependent on the occupation of the zinc binding site, however, a variety of metals appear to support catalysis (23,24). The two metal ions in each At5g18200.1 protein subunit were assumed to be zinc based on their coordination and anomalous signal.

One of the zinc sites is identical to the zinc site found in GalT, directly adjacent to the active site. Figure 6 depicts the coordination of the zinc ion in the At5g18200.1 enzyme and illustrates its possible role in catalysis. As originally seen and described in the *E. coli* GalT structure (25), the zinc appears to be positioning the carbonyl of His184 within hydrogen bonding

distance of the ND1 nitrogen of His186. This may be critical for stabilizing the nucleotidylated intermediate and allowing the reaction to proceed.

The second zinc atom is located in a different position than the iron atom from GalT, although they share one liganding sidechain. The side chain of His310 in the At5g18200.1 enzyme is oriented 180° away from its conserved sequence counterpart in GalT, subsequently shifting the metal binding site 12.5 Å away from the dimer interface, where it is located in GalT, up towards the surface of the protein. It was proposed that the iron atom in GalT may play a role in stabilizing the dimer interface (22), but that seems unlikely with the At5g18200.1 protein.

Enzyme Activity

The ADP-glucose phosphorylase activity of the At5g18200.1 enzyme is the same as that described forty years ago for an ADP-glucose:orthophosphate adenylyltransferase in extracts of wheat germ (26). The wheat germ enzyme was partially purified and shown to accept ADP-1' α -D-glucose, 2'-deoxy-ADP-1' α -D-glucose, and ADP-1' β -D-glucose as adenylyl group donors; however, it was not purified to homogeneity. Both the At5g18200.1 enzyme and the wheat germ adenylyltransferase display the activity corresponding to the systematically named ADP: α -D-aldose-1-phosphate adenylyltransferase (EC 2.7.7.36). No information about the kinetic or chemical mechanism is available for the wheat germ enzyme.

Production of glucose-1-P without pyrophosphate (which is required by the enzyme ADP-glucose pyrophosphorylase) has been reported for several plant extracts, including barley and *Arabidopsis* (27). In the above study, the authors isolated and purified a 35 kDa enzyme from barley leaves that they identified as an ADP-glucose pyrophosphatase, catalyzing the hydrolysis of ADP-glucose into glucose-1-P and AMP. Addition of phosphate was shown to competitively inhibit the enzyme. No sequence was given for the barley enzyme, but it seems unlikely that this protein is identical to the At5g18200.1 enzyme despite the possibility that they serve a similar role in regulating starch production.

A partially purified adenylyltransferase from *Euglena gracilis* catalyzes the reaction of ADP-5'-ribose with phosphate to form ADP and ribose-1,5-bisphosphate (28). This enzyme catalyzes the exchange ADP/ P_i in the absence of ribose-1,5-bisphosphate, apparently by the mechanism of eq. 3. The *Euglena* adenylyltransferase does not accept ADP-glucose as a substrate, so it cannot be the same as the enzyme described here, although the reaction mechanisms may be similar. However, the At5g18200.1 enzyme efficiently catalyzes the phosphorolysis of ADP-5'-ribose (data not shown).

The activity of the At5g18200.1 enzyme is analogous to previously reported nucleotide sugar phosphorolysis activities of enzymes displaying substrate preferences for GDP-mannose and UDP-glucose (29). These enzymes also catalyze exchange reactions such as UDP/ P_i analogous to the ADP-glucose/glucose-1-P exchange described here for the At5g18200.1 enzyme.

Sequence Analysis

A series of BLAST searches using the entire At5g18200.1 enzyme sequence, as well as a portion of the sequence corresponding to the enzyme's adenine binding region, resulted in the identification of a number of plant and bacterial enzymes which may be functionally similar to At5g18200.1. A partial sequence alignment containing a representative sample of these enzymes (Figure 7) reveals a number of residues that may prove useful in distinguishing between uridylyltransferases and adenylyltransferases. All of these enzymes are annotated as putative galactose-1-P uridylyltransferases with the exception of those from *E. coli* and *Homo sapiens*, which are confirmed galactose-1-P uridylyltransferases, and the enzyme from *Thiobacillus denitrificans* which is annotated as an adenylylsulfate:phosphate transferase.

The At5g18200.1 enzyme residues that appear to be unique for adenylyltransferases include Phe65, which is involved in stabilizing the adenine ring through stacking interactions. Residues homologous to Glu70, Trp87, and Arg90 are also strongly conserved. These residues correspond to a region of the At5g18200.1 enzyme that is structurally distinct from the GalT of *E. coli*. Specifically, an extended loop between helix 2 and strand C makes additional contacts with the adenine group (Figure 1). The sidechains of Glu70, Trp87, and Arg90 are involved in hydrogen bonding interactions that may help stabilize this extended loop. Asp98 (*E. coli*) is a unique, strongly conserved residue in GalT enzymes. This residue coordinates the uridine group in the *E. coli* structure (25), but is converted to either a leucine or lysine in the putative adenylyltransferase sequences.

We do not have enough data to distinguish between enzymes with adenylylsulfate:phosphate transferase activity and enzymes with ADP-glucose phosphorylase activity through sequence alignments. The glucose binding region would be the most likely place to distinguish between these activities as the adenylylsulfate:phosphate transferase would have no need to bind glucose. The sequences of the glucose binding regions of potential adenylyltransferases and uridylyltransferases are surprisingly different, however, many of the interactions with glucose in the *E. coli* GalT structure involved backbone atoms (30). Structural alignment of GalT and the At5g18200.1 protein reveal that the positions of the relevant backbone atoms are in identical positions despite the low sequence conservation. Of the five residues in GalT with sidechains coordinated to glucose, four are conserved in every sequence including that of adenylylsulfate:phosphate transferase. As a result, it is difficult to distinguish between adenylylsulfate:phosphate transferases and ADP-glucose phosphorylases. In addition, all of the adenylyltransferases have two cysteine and two histidine residues corresponding to those involved in coordinating the second zinc atom in the At5g18200.1 enzyme, and all are missing one or more residues needed for coordinating iron as in the *E. coli* GalT structure (22).

Possible Role in Starch Synthesis

The biological function of the At5g18200.1 protein cannot be assigned based solely on *in vitro* biochemical properties. The reaction catalyzed represents a means of degrading ADP-glucose in such a way as to conserve the free energy of the phosphoanhydride and aldose-1-phosphate linkages. On this basis, we hypothesize that this enzyme participates in ADP-glucose homeostasis. ADP-glucose is known to play a prominent role in starch metabolism in plants (31). ADP-glucose is synthesized by the enzyme ADPglucose pyrophosphorylase (APGase) and is then polymerized via the soluble and granule-bound starch synthases (32). While most evidence has suggested that these reactions take place entirely in the plastids of non-cereal plants, such as *Arabidopsis*, recent studies have suggested that ADP-glucose may also be synthesized in the cytosol and transported into the plastid (33,34).

The At5g18200.1 enzyme is not currently implicated in this regulation. Also, it is not known under what conditions and in which cellular compartments of *Arabidopsis* the At5g18200.1 enzyme is normally produced. Moreover, although the enzyme is quite active with ADP-glucose and P_i ($k_{cat}/K_m = 0.4 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$ for the reaction of ADP-glucose at saturating P_i), there could be even better substrates. We can assert that whenever this enzyme is present with ADP-glucose, P_i , and Mg_{2+} the ADP-glucose will be converted to ADP and glucose-1-P. Biological experiments, including gene deletions, will be required to deduce the biological function of the *Arabidopsis* At5g18200.1 enzyme.

In conclusion, we have solved the structure of the At5g18200.1 protein and established its structural homology to the *E. coli* GalT. We have shown that the enzyme does not possess GalT activity, but instead displays adenylyltransferase activity. We have further shown that, of the substrates tested, the enzyme displays the greatest activity when ADP-glucose and P_i are used as the donor/acceptor pair, making the enzyme an ADP-glucose phosphorylase, or

ADPGPase. We have determined kinetic and equilibrium constants for this reaction and shown that the reaction proceeds through a bi bi double displacement mechanism. We have also isolated and observed the enzyme-AMP intermediate suggested by this mechanism through X-ray crystallography.

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Figure 1. Ribbon and Topology Diagrams of the At5g18200.1 protein

The figure depicts chain A of the adenylylated monomer. Zinc atoms are shown as grey spheres and the AMP group is shown as a stick model. Both ribbon and topology diagrams are labeled in an identical manner using letters A-N for the β -strands and 1–13 to distinguish the helices. These figures were prepared using Pymol(35) and Topdraw(36).

Figure 2. Dimer interface of the At5g18200.1 protein

The figure depicts the At5g18200.1 dimer along the 2-fold symmetry axis with chain A (yellow and blue) and chain B (cyan and black) in a ribbon representation. Zinc ions are shown as grey spheres and the adenylyl intermediate is shown as a stick model. The active site of each subunit is located along the exterior of the GalT-like half-barrel and positioned near the dimer interface. Arrows specify regions of contact between the two monomers, namely the interchain β -sheets formed by strands A, B, N, and E and α -helix 12. The figure was generated with Pymol(35).

Figure 3. Initial rates for exchange of ^{14}C from ADP- ^{14}C glucose into glucose-1-P
 The initial rates were measured as described in the EXPERIMENTAL PROCEDURE and plotted in double reciprocal form. The ADP- ^{14}C glucose (2.8×10^7 cpm / μmol) concentrations were 0.25 to 1.0 μM and glucose-1-P concentrations were 10 to 400 μM . The velocity units are μM per min with 8.1×10^{-3} μM At5g18200.1 enzyme. The parameters resulting from fitting eq. 1 to the data are listed in Table 3.

Figure 4. Initial rates of glucose-1-P formation in the action of the At5g18200.1 enzyme

Initial rates of glucose-1-P formation at varying ADP- 14 C]glucose concentrations and several fixed P_i concentrations were measured as described in the EXPERIMENTAL PROCEDURE and plotted in double reciprocal form. The ADP- 14 C]glucose concentrations were 0.2 to 7.0 μ M and P_i concentrations were 5 to 60 μ M. The velocity units are μ M per min at 6.7×10^{-3} μ M At5g18200.1 enzyme. The parameters resulting from fitting eq. 1 to the data are listed in Table 3.

Figure 5. Active site of the At5g18200.1 enzyme

The figure illustrates the interactions between the enzyme active site and the AMP-histidine intermediate (magenta) formed upon addition of ADP-glucose. The figure was generated with Pymol(35).

Figure 6. Role of zinc in catalysis

The zinc ion bound by the sidechain of His 184 may be necessary for positioning the mainchain carbonyl oxygen of His 184 2.7 Å away from the ND1 nitrogen of histidine 186 in chain A (2.8 Å in chain B). This interaction may help stabilize the His-AMP intermediate. Zinc is depicted as a grey sphere. The figure was generated with Pymol(35).

<i>Arabidopsis thaliana</i>	65	FCIGREQECAPELFRVP-----DHDPN	WKL	RV	IE	NL	95
<i>Lycopersicon esculentum</i>		FCAGHEHECAPEIFRVPA----DSTND	WKI	RV	IQ	NL	
<i>Solanum tuberosum</i>		FCAGHEHECAPEIFRVPA----DSTND	WKI	RV	IQ	NL	
<i>Vitis vinifera</i>		FCSGHEHECAPEIFRFP----DSTVD	WKI	RV	IQ	NL	
<i>Oryza sativa</i>		FCQGRESECAPEIFRVPA---PDASP	WRI	RV	IE	NL	
<i>Triticum aestivum</i>		FCLGRESECAPEIFRVPPP---DASPP	WRI	RV	IE	NL	
<i>Thermatoga maritima</i>	42	FDYGNHTTPPEIFAIRPADTEPNTPG	WWV	RV	VP	NK	77
<i>Pelobacter propionicus</i>	41	FCYGNEDKTPPEIFAIRPSG-MPNAAN	WRV	RV	IP	NK	75
<i>Thiobacillus denitrificans</i>	54	FCRGNEDKTPPAI	LWPES-----ADWQI	RM	VE	NL	83
<i>Escherichia coli</i>	54	LCAGNVRVTGDKNP	PDYT-----GT	YV	FT	ND	78
<i>Homo sapiens</i>	74	LCPGAIRANGEVNPQYD-----ST	FL	FD	ND		98
<i>Arabidopsis thaliana</i>	167	YIQVFKNQGASAGASMSHSHSQMMAL	PV	VP	PT	VSSR	202
<i>Lycopersicon esculentum</i>		YVQVFKNHGASAGASMSHSHSQMIAL	P	I	V	P	TVSAR
<i>Solanum tuberosum</i>		YVQVFKNHGASAGASMSHSHSQMIAL	P	I	V	P	TVSAR
<i>Vitis vinifera</i>		YIQVFKNHGASAGASMSHSHSQIMAL	P	I	V	P	TVSSR
<i>Oryza sativa</i>		TGMVFNHGASAGASMAHSHSQMLGT	P	F	V	P	PSVTR
<i>Triticum aestivum</i>		YVQVFKNQGASAGASMAHSHSQMLGT	P	F	V	P	PSVTSR
<i>Thermotoga maritima</i>	147	YILIFKNHGRDAGASLSHPSHQIIAL	P	I	M	P	KRVQEE
<i>Pelobacter propionicus</i>	145	YMVIFKNHGLRAGATLHSHSqliAV	P	L	L	P	PVAATE
<i>Thiobacillus denitrificans</i>	155	YVLVFKNFGPAAGASIPHTSQVIAM	P	V	V	P	ENVEAE
<i>Escherichia coli</i>	147	WVQVFENKGAAMGCSNPHPHGQIWAN	S	F	L	P	NEAERE
<i>Homo Sapiens</i>	147	WVQIFENKGAMGCSNPHPHCQVWAS	S	F	L	P	DIAQRE

Figure 7. Portion of sequence alignment between uridylyltransferases and putative adenylyltransferases

The At5g18200.1 enzyme as well as all putative adenylyltransferases from plant species are labeled green. Putative adenylyltransferases from bacteria are labeled red. Sequence labels for confirmed uridylyltransferases are colored blue. Residues that appear to distinguish adenylyltransferases from uridylyltransferases are colored orange. The top block of sequences correspond to the adenine/uridine binding region. The lower block corresponds to the region that contains the HXHXQ active site motif. The start codon for some of the sequences was unclear and subsequently no residue numbering was included. The alignment was created with ClustalW(37). The sequence codes for the sequences included in the alignment are as follows: NP_197321 (Genbank), TC156309 (TIGR), TC113581 (TIGR), TC40775 (TIGR), 9635.m00702 (Plasmid Proteome Database), TC237707 (TIGR), NP_228704 (Genbank), EA03679 (Genbank), AAF64398 (Genbank), P09148 (UniProt), and P07902 (UniProt).

Summary of crystal parameters, data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

Table 1

Spacegroup	Native	Selenium1	Selenium2	Adenylated-Native
Unit Cell Parameters (Å)	P2 ₁ -2 ₁ -1			
Collection and Phasing Statistics	a=60.0 b=95.5 c=110.5			a=62.0 b=95.7 c=110.9
Wavelength	0.97858	0.97908	0.97908	0.97948
Resolution Range (measured/unique)	27.59-2.30 (2.35-2.30)	46.99-3.20 (3.27-3.20)	48.57-3.20 (3.27-3.20)	34.49-1.83 (1.90-1.83)
Completeness (%)	177844/27575	153206/20251	150421/20137	399212/57501
R_{merge}	95.6 (80.4)	99.8 (100.0)	99.7 (100.0)	96.5 (71.4)
R_a	0.044 (0.406)	0.107 (0.284)	0.099 (0.305)	0.083 (0.386)
Redundancy	6.4 (5.1)	7.6 (7.8)	7.5 (7.8)	6.9 (5.5)
Mean $I/\sigma(I)$	24.03 (3.65)	16.95 (8.42)	17.20 (7.88)	15.24 (4.54)
Refinement Statistics				
Resolution Range (Å)	27.59-2.30			31.28-1.83
(total/test)	25918/2581			54539/2903
R_{cryst}	0.215			0.186
R_{free}	0.269			0.231
R.m.s.d. bonds (Å)	1.3			1.558
R.m.s.d. angles (°)	0.006			0.017
Average protein factor (Å ²)	47.30			24.00
Average solvent factor (Å ²)	43.49			32.86
Average substrate factor (Å ²)				25.27

^a $R_{merge} = \sum h \sum i |I_i(h) - \langle I(h) \rangle| / \sum h \sum i I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

^b $R_{cryst} = \sum h ||F_{obs} - F_{calc}|| / \sum h |F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

^c R_{free} was calculated as R_{cryst} using 5.0% of the randomly selected unique reflections that were omitted from structure refinement.

Table 2

Nucleotidyl donor activities of the At5g18200.1 enzyme.

Donor substrate	Acceptor substrate	Activity ($\mu\text{mole min}^{-1} \text{ mg protein}^{-1}$) ^a
UDP-glucose ^b	galactose-1-P ^c	0.0097
ADP-glucose ^d	galactose-1-P ^e	0.086
ADP-glucose ^d	mannose-1-P ^c	0.24
ADP-glucose ^d	P _i ^f	7.1
ADP-glucose ^d	MgPP _i ^g	none detected

^a Rates of glucose-1-P production measured at pH 8.5 in sodium bicarbonate buffer at 27 °C as described in the Experimental section.^b 50 μM ^c 200 μM ^d 20 μM ^e 250 μM ^f 31 μM ^g 100 μM

Table 3

Kinetic parameters for reactions of the At5g18200.1 enzyme.

Reaction	k_{cat} (s^{-1})	$K_{\text{m}}^{\text{ADP-glucose}}$ (μM)	K_{m}^{P} (μM)	$K_{\text{m}}^{\text{Glucose-1-P}}$ (μM)
Overall ^a	2.7 ± 0.4	6.9 ± 1.2	90 ± 19	- -
Exchange ^b	4.1 ± 0.3	1.4 ± 0.2	- -	83 ± 8

^aReaction of ADP-glucose with P_i to form ADP and Glucose-1-P at pH 8.5 and 25 °C.^bExchange of ¹⁴C from ADP[¹⁴C]Glucose into Glucose-1-P at pH 8.5 and 25 °C.