The higher plant ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) is a key enzyme in the control of starch synthesis in higher plants. The higher plant enzyme has a tetrameric structure consisting of two distinct subunit types, large subunit (LS) and small subunit (SS) (1–4). These subunit types share considerable sequence identity (∼53%) and similarity (∼73%) indicating that both subunits originated from a common gene ancestor which has diverged at different rates over time (5–7). When expressed in bacteria and readily forms inclusion bodies. Hence, all available evidence on the role of LS in enzyme function was obtained by studying its operability with the SS. Site-directed mutagenesis studies on conserved residues predicted to bind to effectors and substrates in the potato tuber subunits, suggest that the SS is catalytic and regulatory, whereas the LS lacked these properties and simply modulates the regulatory activity of the SS by protein-to-protein interactions (18, 19). An alternative view on subunit roles in enzyme function was obtained by the study of potato heterotetrameric enzymes formed by different combinations of wild-type and mutant LSs and SSs (9). Such results showed that the net allosteric properties of the heterotetrameric enzyme is contributed by both subunits and is a product of synergy between LS and SS interactions. A similar conclusion for a regulatory role for the LS was also made for the maize endosperm AGPase (10). These observations suggest that the SS is catalytic and regulatory, whereas the LS lacks these properties.

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The LS of the potato tuber AGPase is also likely to significantly participate in catalysis by affecting the capacity of the heterotetrameric enzyme to bind substrates and effectors (6).
Substantial evidence for a catalytic role, albeit indirect, for the LS was obtained by identification of a substrate binding site for ATP by photoaffinity labeling studies with 8-NN-ATP (21). Mutations of selected residues located within ATP binding site of the LS significantly altered the catalytic properties of the AGPase heterotetramer.

Based on homology studies of other structurally related sugar nucleotide pyrophosphorylases (22–24), the metal-binding Asp143 (originally assigned as Asp145) of the potato AGPase SS was suggested to be essential for catalytic activity, a prediction verified by site-directed mutagenesis of this residue, which lowered catalytic rate more than four orders of magnitude compared with the wild-type enzyme. A similar mutation in the conserved Asp158 of the LS, however, reduced catalysis only 1.5- to 2.6-fold (19). Our recent result, however, showed that the replacement of Asp158 by Leu lowered catalytic rates by 11-fold and catalytic efficiencies by 23- to 26-fold depending on the substrates of the enzyme (21), indicating the Asp158 residue in the LS is important for enzyme catalysis. Interestingly, introduction of a single residue replacement, T51K, or double mutations, K41R and T51K, into the wild-type LS resurrected partially (5% of wild-type activity) the catalytic activity of the heterotetrameric enzyme containing the catalytically silenced S3143N (7). Overall, these results, together with those obtained from earlier studies on substrate and effector binding, indicate that the LS is catalytically inefficient but has many of the necessary elements for this function.

Although available evidence indicates that the LS, which is capable of binding substrates (6, 18) and effectors (25), is catalytically defective, direct evidence for this property has yet to be obtained as all available evidence in support of this view is deduced from studies of the heterotetrameric enzyme forms. To further enhance our understanding of the role of LS in enzyme function, we identified a mutant LS containing a S302N replacement, which significantly elevated solubility of the LS and enabling assembly and formation of LS homotetramer. Results from biochemical and kinetic analysis of LS homotetramer and heterotetrameric forms with catalytic-silenced S3143N showed that the LS in the absence of SS displays very low catalytic activity and is allosteric-insensitive. When operating with the SS, however, catalysis by the LS is stimulated, and allosteric regulatory properties of the LS are unmasked.

**EXPERIMENTAL PROCEDURES**

**Materials**—[14C]Glucose 1-phosphate and [32P]pyrophosphate were purchased from ICN Pharmaceuticals and PerkinElmer Life Sciences, respectively. Radioactive 8-azidoadenosine 5'-[α-32P]triphosphate ([α-32P]8-NN-ATP) and nonradioactive 8-NN-ATP were purchased from Affinity Labeling Technologies, Inc. Reagents including ATP, Glc 1-P, and ADP-glucose were obtained from the Sigma-Aldrich and were of analytical grade or higher.

**Expression and Purification of the AGPase Proteins**—The wild-type and various LS mutants were expressed in E. coli EA345 cells by transforming the pRARE plasmid isolated from Rosetta™ cell (Novagen) into EA345 cell. For purification of the SS homotetramer the His6-tagged S3143N protein (26) was expressed in EA345 cells. AGPases were purified as described previously using a Bio-Logic DuoFlow Chromatography system (Bio-Rad) (6, 21) with minor modifications. Briefly, the active AGPase fractions obtained from a DEAE-Sepharose FF (Amersham Biosciences) chromatography and TALON™-IMAC (Clontech Lab) were subjected to POROS 20 HQ (PerSeptive Biosystems) column chromatography. Aliquots of the purified enzyme preparation were stored frozen in liquid nitrogen and shelved at −80 °C until used for analysis.

**AGPase Assay and Kinetics**—AGPase activities were assayed in the pyrophosphorylase direction (Assay A) during enzyme purification and in the ADP-glucose synthesis direction (Assay B) for kinetic characterization (6). Unless stated, saturating amount of substrates was used for both methods. One enzyme unit is defined as 1 micromole of ATP (Assay A) or ADP-glucose (Assay B) formed for 1 min at 37 °C. KaleidaGraph 3.5 (Synergy software) was used to fit the experimental data to the modified Hill equations (6). The kinetic values (S0.5, kcat, and kcat/S0.5) were determined as described previously (6).

**Chemical and Site-directed Mutagenesis**—200 μg of plasmid DNA (pSH345 or pSH274 containing LK41R and K41R) were subjected to chemical mutagenesis by incubating at 37 °C for 20 h in 4 ml of 0.1 M sodium phosphate (pH 6.0), 0.8 M hydroxylamine-HCl, and 1 mM EDTA (27). After neutralization with 400 μl of 1.5 M Tris-HCl (pH 8.8) the plasmid DNAs were then precipitated in 80% (v/v) ethanol. The plasmid DNAs were then used to transform E. coli EA345 cells expressing S302N. Cells were grown on NZCYM media supplemented with 100 mg/liter ampicillin and 50 mg/liter kanamycin with 0.4% (v/v) glycerol as the carbon source, which readily allows the efficient induced expression of both subunits from the lac promoters (6). After overnight culture at 37 °C, the bacterial colonies were screened for glycogen production by exposure to iodine vapor. Plasmid DNAs expressing the LS mutants were purified and were subjected to DNA sequencing to identify changes in nucleotide sequence.

Site-directed mutagenesis of the AGPase subunit sequence was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene) as described previously (21): LS302N-R, 5′-AAATCGTTTTATAATGCTaacTTGGCACT-3′ and LS302N-R, 5′-CTCTTGTGATGTCACAGTGTTTATACATTATAAAACGATT-3′ (target sequences are in lowercase).

**Labeling of AGPases with 8-Azido-adenosine 5′-Triphosphate**—The purified AGPases were reduced, desalted, and labeled as described previously (6, 21).

**Modeling of AGPase Structures**—DeepView, the Swiss-PdbViewer was used for modeling of the three-dimensional structure of the LS based on the crystal structure of the potato SS homotetramer (28) as the template scaffold. Coordinates for the AGPase structures (1yp3) were retrieved from RCSB Protein Data Bank. Pov-Ray was used for rendering the structure.
Potato Tuber AGPase Large Subunit Homotetramer

A

NaCl concentration (M)

0

0.15

0.3

Optical density

220 (nm)

LRKSSi

LRKNSi

Elution volume (ml)

0

20

40

B

CBB

Anti-LS

Anti-SS

FIGURE 1. Purification profiles of various AGPases. AGPase proteins were initially purified on DEAE-Sepharose FF chromatography followed by TALON™-immobilized affinity chromatography. The resulting enzyme preparation was then loaded onto a POROS 20HQ column (HR-5/5, bed volume, 1 ml) and washed with 0.15 M NaCl and eluted with 0.3 M NaCl (A) at the flow rate of 2 ml/min. Fractions containing proteins were analyzed by SDS-PAGE and immunoblot analysis (B) using antibodies raised against the potato LS (53 kDa) or SS (50 kDa). For convenience abbreviations were used for various mutations: R for K41R; K for T51K; N for S302N; and S for D143N. CBB, Coomassie Brilliant Blue R-250.

Protein Analysis—Protein concentration was measured using the Advanced Protein Assay Reagent from Cytoskeleton (Denver, CO) with bovine serum albumin (fraction V) as the standard. SDS-PAGE was performed as described in a previous study (26). Immunoblot analysis was performed using anti-potato AGPase LS or anti-potato AGPase SS as described before (6).

RESULTS

Substitution of Ser^{302} with Asn in the LS Significantly Enhanced Glycogen Production in Bacterial Host Cells—To provide further insights on the role of the potato tuber LS in AGPase function, the expression plasmid DNA containing His_{6}-tagged L^K41R-T51K (abbreviated L^RK) was subjected to chemical mutagenesis (supplemental Fig. S1A) and co-expressed with the catalytic-silenced S^{D143N} (abbreviated S^{Si}) mutant (7, 21) in bacterial cells lacking AGPase activity. These cells were then analyzed for glycogen production by exposure to iodine vapor. Under these conditions, cell expressing L^{WTSSi} are devoid of glycogen and do not stain, whereas cells expressing L^{LKSSi} enzyme stain lightly as they accumulate small but readily detectable levels of glycogen due to the low catalytic activity of this enzyme (supplemental Fig. S1B). More than 6 × 10^9 bacterial colonies derived from the mutagenized potato tuber AGPase LS were examined by iodine staining resulting in the identification of 26 colonies, which exhibited significantly enhanced glycogen accumulation (supplemental Fig. S1B). Interestingly, sequence analysis showed that all 26 LS sequences obtained from these excess glycogen accumulating cells contained a common third mutation, S302N, in addition to the pre-existing K41R and T51K mutations (for brevity, LS containing these three mutations will be denoted as L^{RKN}). Six plasmids contained silent mutations in addition to S302N, whereas a single plasmid contained a fourth mutation V159I in LS. Thus, addition of S302N mutation in the LS enhances net AGPase activity which, in turn, increases glycogen production.

The S302N Mutation Dramatically Increases LS Solubility—Heterotetrameric forms of the L^{LKSSi} and mutant L^{RKNSSi} enzymes were purified to near homogeneity (>95%) by multiple chromatography steps. During the final clean-up of the enzyme activity using a strong anion-exchange chromatography step, an anomalous protein elution profile was observed for the L^{RKNSSi} enzyme. Typically, the wild-type AGPase elutes from the anion-exchange column as a single major protein peak at 0.3 M NaCl with very little protein eluting at lower salt concentrations (21). Similar elution profile was also observed for the L^{RKSNSi} enzyme (Fig. 1A). Although much of the L^{RKNSSi} enzyme also eluted at 0.3 M NaCl, a prominent peak was also observed eluting at 0.15 M NaCl (peak a of Fig. 1A, lower panel). Results from SDS-PAGE and immunoblot analysis showed that the 0.15 M NaCl fraction contained only the LS (Fig. 1B), indicating that a significant amount of L^{RKN} was unassembled with the SS and remained soluble throughout the enzyme purification steps. Analysis of the 0.15 M NaCl protein fraction by Superdex-200 gel filtration chromatography indicated that the L^{RKN} eluted with a molecular size of 223 kDa (supplemental Fig. S2). Hence, the soluble L^{RKN} readily self-assembles into a homotetrameric form. Further studies showed that the soluble L^{N} also readily self-assembles into a 220 kDa oligomer (supplemental Fig. S2).

S302N Mutation Is Responsible for the Enhanced Solubility and Formation of the Potato LS Homotetramer—To determine whether the Asn^{302} residue was responsible for the increase in solubility of the potato tuber LS we compared the total and soluble expression of various LSs containing or lacking this mutation. When expressed in E. coli cells alone without the SS counterpart, all of the various LS types were expressed at nearly equal levels when total cell extracts were examined by SDS-PAGE (supplemental Fig. S3A). When only the soluble fractions were examined, however, only the LS forms containing S302N were found at substantial levels while the same LS forms lacking S302N were present at very low levels indicating that the bulk of the expressed LS was insoluble (supplemental Fig. S3, B and C). Quantitative analysis of the soluble forms of the homotetramers after enzyme purification showed that L^{RKN} and L^{N} were 376- and 77-fold more soluble than L^{LK} and wild-type L^{WT} respectively (supplemental Table S1). These observations indicate that introduction of an Asn residue at position 302 is responsible for the enhanced solubility of the potato tuber LS.

The LS Homotetramer Is Not Affected by Allosteric Effectors—Examination of the enzymatic activity of purified L^{N} homotet-
rameric protein showed that it had very low but measurable activity (~0.4 unit/g). This activity was 93-fold less than that measured for the homotetramer \( S^{WT} \) (37 units/g) when assayed in the absence of the activator 3-PGA (Table 1). AGPase activity was elevated >800-fold by the introduction of K41R and T51K substitutions into LN to yield LRKN (330 units/g).

To determine whether the relatively low catalytic activity of LN and LRKN homotetramers was due to its dependence on allosteric activation, the enzyme activity was assayed over a broad range of 3-PGA concentrations. Interestingly, unlike the

### TABLE 1

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Large</th>
<th>Small</th>
<th>3-PGA, ( A_{0.5} )</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L^{RKN} )</td>
<td>( S^W )</td>
<td>( 25 \pm 1 )</td>
<td>(0.8)</td>
<td>( 63 \pm 10 )</td>
</tr>
<tr>
<td>( L^{RKN} )</td>
<td>( S^L )</td>
<td>( 6 \pm 1 )</td>
<td>(0.9)</td>
<td>( 48 \pm 5 )</td>
</tr>
<tr>
<td>( L^N )</td>
<td>( S^W )</td>
<td>( 78 \pm 0.7 )</td>
<td>(0.7)</td>
<td>( 4.3 \pm 0.1 )</td>
</tr>
<tr>
<td>( L^{RKN} )</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>( L^N )</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>( S^{WT} )</td>
<td>10400</td>
<td>3.80</td>
<td>3.80</td>
<td>3.80</td>
</tr>
<tr>
<td>( L^{WT} )</td>
<td>( S^W )</td>
<td>90</td>
<td>11</td>
<td>1.5</td>
</tr>
<tr>
<td>( L^{RKN} )</td>
<td>( S^W )</td>
<td>109</td>
<td>15</td>
<td>1.6</td>
</tr>
<tr>
<td>( L^N )</td>
<td>( S^W )</td>
<td>89</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>( L^{RKN} )</td>
<td>( S^W )</td>
<td>79</td>
<td>2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Specific activity was obtained at saturated concentrations of 3-PGA except that 0.5 mM 3-PGA was used to measure activity of LRKN and LN.

* Fold activity = maximal specific activity in the presence of saturated concentration of 3-PGA-specific activity in the absence of 3-PGA.

* n/c = non-calculable due to lack of activation by 3-PGA (LRKN) or very low enzyme activity (LN and SS).

Inefficient—The LRKN homotetramer showed significantly low affinity toward ATP and Glc-1-P compared with the heterotetrameric enzyme forms (Table 2). The \( S_{0.5} \) values for ATP were 1927 \( \mu M \) (\( n_H = 1.9 \)) in the presence of 0.5 mM 3-PGA and 1880 \( \mu M \) (\( n_H = 1.6 \)) in the absence of 3-PGA. The \( S_{0.5} \) values for Glc-1-P were 4307 or 4175 \( \mu M \) (\( n_H = 1.3 \) or 1.4) in the presence or absence of 0.5 mM 3-PGA. When kinetic study was done in the pyrophosphorylase direction, similar trends in \( S_{0.5} \) values were obtained for ADP-glucose and PP, with the LRKN homotetramer having \( S_{0.5} \) values of 3- to 5-fold and 2- to 3-fold higher than \( L^{RKN}S^{WT} \), respectively (Table 3). These results also indicate that the catalytic properties of the LS mutant are not affected by 3-PGA, and the LS mutant is less efficient at substrate binding than the enzyme forms containing SS.

**The LS Mutant Homotetramer Is Catalytically Very Inefficient**—The LRKN homotetramer showed significantly low affinity toward ATP and Glc-1-P compared with the heterotetrameric enzyme forms (Table 2). The \( S_{0.5} \) values for ATP were 1927 \( \mu M \) (\( n_H = 1.9 \)) in the presence of 0.5 mM 3-PGA and 1880 \( \mu M \) (\( n_H = 1.6 \)) in the absence of 3-PGA. The \( S_{0.5} \) values for Glc-1-P were 4307 or 4175 \( \mu M \) (\( n_H = 1.3 \) or 1.4) in the presence or absence of 0.5 mM 3-PGA. When kinetic study was done in the pyrophosphorylase direction, similar trends in \( S_{0.5} \) values were obtained for ADP-glucose and PP, with the LRKN homotetramer having \( S_{0.5} \) values of 3- to 5-fold and 2- to 3-fold higher than \( L^{RKN}S^{WT} \), respectively (Table 3). These results also indicate that the catalytic properties of the LS mutant are not affected by 3-PGA, and the LS mutant is less efficient at substrate binding than the enzyme forms containing SS.

**The LS Mutant Homotetramer Binds ATP But Less Efficiently**—Our previous studies (6, 21) have demonstrated that both the LS and SS in the heterotetrameric form are labeled at equivalent rates with 8-\( N^p \)-ATP, an analog which can readily substitute for ATP in catalysis. To determine the LS binding properties for ATP, \( L^{RKN} \) and \( L^N \) homotetramers were incubated with increasing concentrations of radioactive 8-\( N^p \)-ATP analog (Fig.
Potato Tuber AGPase Large Subunit Homotetramer

4) using LRKNS\textsuperscript{WT} and S\textsuperscript{WT} as the controls. The labeling patterns obtained showed that irrespective of enzyme activity (L\textsuperscript{RK} = 330 units/g and L\textsuperscript{N} = 0.4 unit/g) both LS homotetramers were photoaffinity-labeled with the ATP analog at similar efficiencies: ATP labeling constants (K\textsubscript{i}) for LRKN and LN were estimated to be 590 M\textsuperscript{-1} and 530 M\textsuperscript{-1}, respectively. The labeling constant for L\textsuperscript{RK} was 3.3- and 2.5-fold higher than those obtained for the S\textsuperscript{WT} homotetramer (K\textsubscript{i} = 180 M\textsuperscript{-1}) and LRKNS\textsuperscript{WT} heterotetramer (K\textsubscript{i} = 230 M\textsuperscript{-1}), respectively. Moreover, unlike the similar affinity values obtained by photoaffinity labeling (K\textsubscript{i}) and enzyme kinetics (S\textsubscript{0.5}) obtained for the S\textsuperscript{WT} and L\textsuperscript{RKNS\textsuperscript{WT}} forms, the K\textsubscript{i} values of the LS homotetramers were ~3- to 4-fold lower than the apparent ATP S\textsubscript{0.5} for L\textsuperscript{RK}.

This significant disparity indicates that the apparent S\textsubscript{0.5} for ATP for the L\textsuperscript{RK} homotetramer overestimates the actual binding efficiency of ATP likely due to its poor catalytic activity by the LS. Moreover, it demonstrates that the K41R and T51K replacements alter the catalytic rate and not the substrate binding properties.

The LS Mutant Homotetramer Is Less Heat-stable—Heat stabilities of the LS mutant homotetramer (L\textsuperscript{RK}), SS homotetramer (S\textsuperscript{WT}), and their combined heterotetramer (LRKNS\textsuperscript{WT}) were examined by incubating the enzymes at 30 °C, 37 °C, 45 °C, 52 °C, and 60 °C for 5 min. The enzyme activity remaining was measured at saturated concentrations of substrates and 3-PGA (Fig. 5). At 60 °C the L\textsuperscript{RKNS\textsuperscript{WT}} heterotetramer retained 92% of AGPase activity and the S\textsuperscript{WT} homotetramer had an intermediate level of activity (68%), whereas the L\textsuperscript{RK} showed only <7% of activity. This result indicates that the homotetrameric

![FIGURE 3. Phosphate inhibition profiles of the AGPases.](http://www.jbc.org/)

Reactions were performed in the forward (ADP-glucose synthesis) direction under saturating substrate conditions and with 0.5 mM 3-PGA (see legend of Table 1). The smooth fit feature of KaleidaGraph 3.5 was used for curve fitting. L\textsuperscript{RK} (c), S\textsuperscript{WT} (d), and L\textsuperscript{RKNS\textsuperscript{WT}} (e).

### TABLE 2

Catalytic properties of the various AGPases in the ADP-glucose synthesis (forward) direction

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Large</th>
<th>Small</th>
<th>ATP</th>
<th>Glc-1-P</th>
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</thead>
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<tr>
<td></td>
<td>S\textsubscript{0.5}</td>
<td>k\textsubscript{cat}</td>
<td>C.E.</td>
<td>S\textsubscript{0.5}</td>
</tr>
<tr>
<td>LRKN</td>
<td>142 ± 9</td>
<td>121 ± 4</td>
<td>857 ± 24</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>158 ± 8</td>
<td>14 ± 1</td>
<td>745 ± 22</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>175 ± 23</td>
<td>165 ± 5</td>
<td>982 ± 30</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>192 ± 12</td>
<td>89 ± 2</td>
<td>940 ± 22</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>200 ± 14</td>
<td>123 ± 8</td>
<td>614 ± 19</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>269 ± 12</td>
<td>58 ± 1</td>
<td>217 ± 9</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>399 ± 30</td>
<td>113 ± 6</td>
<td>283 ± 6</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>446 ± 26</td>
<td>145 ± 2</td>
<td>327 ± 16</td>
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<tr>
<td>LRKN</td>
<td>701 ± 39</td>
<td>154 ± 1</td>
<td>233 ± 7</td>
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<tr>
<td>LRKN</td>
<td>718 ± 70</td>
<td>159 ± 2</td>
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<tr>
<td>LRKN</td>
<td>735 ± 75</td>
<td>159 ± 2</td>
<td>247 ± 7</td>
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<tr>
<td>LRKN</td>
<td>752 ± 80</td>
<td>159 ± 2</td>
<td>247 ± 7</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>LRKN</td>
<td>769 ± 85</td>
<td>159 ± 2</td>
<td>247 ± 7</td>
<td>1 (1.0)</td>
</tr>
</tbody>
</table>

* Relative catalytic efficiency of AGPase variant with respect to L\textsuperscript{RKNS\textsuperscript{WT}}.

* Relative catalytic efficiency of AGPase variant with respect to L\textsuperscript{RKNS\textsuperscript{WT}}.

** Relative catalytic efficiency of AGPase variant with respect to L\textsuperscript{RKNS\textsuperscript{WT}}.

### TABLE 3

Catalytic properties of the various AGPases

Activity was determined in the reverse pyrophosphorolysis direction in the presence of saturating concentrations of substrates: 2 mM pyrophosphate and 10 mM ADP-glucose for L\textsuperscript{RK} homotetramer, and 1.5 mM pyrophosphate and 2 mM ADP-glucose for the rest of the enzymes. Concentrations of 3-PGA used in the assays were 0.5 mM for L\textsuperscript{RK} homotetramer, 5 mM for the various AGPase heterotetramers, and 10 mM for S\textsuperscript{WT} homotetramer. The S\textsubscript{0.5} value corresponds to the substrate concentration (\textmu M) required for 50% maximal activity of enzyme. n\textsubscript{H}, Hill coefficient. C.E.: catalytic efficiency (k\textsubscript{cat}/S\textsubscript{0.5}) \times 10\textsuperscript{4}.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Large</th>
<th>Small</th>
<th>ADP-glucose</th>
<th>Pyrophosphate</th>
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<tr>
<td></td>
<td>S\textsubscript{0.5}</td>
<td>k\textsubscript{cat}</td>
<td>C.E.</td>
<td>S\textsubscript{0.5}</td>
</tr>
<tr>
<td>LRKN</td>
<td>194 ± 19</td>
<td>139 ± 11</td>
<td>717 ± 15</td>
<td>1 (10)</td>
</tr>
<tr>
<td>LRKN</td>
<td>585 ± 20</td>
<td>131 ± 4</td>
<td>232 ± 47</td>
<td>(0.3)</td>
</tr>
<tr>
<td>LRKN</td>
<td>1843 ± 98</td>
<td>72 ± 0</td>
<td>39 ± 0.3</td>
<td>(0.00005)</td>
</tr>
<tr>
<td>LRKN</td>
<td>1927 ± 92</td>
<td>70 ± 0</td>
<td>39 ± 0.3</td>
<td>(0.00005)</td>
</tr>
<tr>
<td>LRKN</td>
<td>19 (1.3)</td>
<td>172 ± 0</td>
<td>82 ± 5</td>
<td>486 ± 29</td>
</tr>
</tbody>
</table>
The LS Mutant Homotetramer Is Not Redox-regulated—Under non-reducing conditions, the various heterotetrameric AGPases, including wild-type $L^{WT}S^{WT}$ (Fig. 7A) and mutants $L^{RKN}S^{WT}$ and $L^{NS}S^{WT}$ (Fig. 7B), showed that a significant proportion of the SSs were linked by an interchain disulfide bond. This condition was also the case for the $S^{WT}$ homotetramer (Fig. 7D). Treatment of these enzymes with DTT resulted in the disappearance of the SS dimer (100 kDa). Under the same conditions, the LS remained as a single subunit. Under the same conditions, the LS remained as a single subunit.

We also examined the effects of the reducing agent (DTT) together with ADP-glucose on the regulatory properties of various AGPase heterotetramers and homotetramers (supplemental Table S2 and Fig. S5). Consistent with results from previous reports (12, 17), the reduced form of the wild-type AGPase heterotetramer showed higher activation at lower concentrations of 3-PGA than its non-reduced form. The same trends were observed for the other mutant heterotetramers. The increased activation was most remarkable for the $S^{WT}$ homotetramer. However, the reductive activation was not observed for $L^{RKN}$ homotetramer. In addition, the substitutions (K41R, T51K, and/or S302N) in the LS did not significantly affect the redox regulation (supplemental Fig. S5). Collectively, these results suggest that the LS is not directly involved in the redox regulation of the potato tuber AGPase.

**DISCUSSION**

The exact role of the LS in the functioning of the heterotetrameric AGPase from potato tuber remains largely unresolved as unlike the SS, which is capable of forming a catalytically active homotetrameric enzyme, the LS is largely insoluble and forms inclusion bodies when expressed as a recombinant form in bacterial cells. Although crude insect cell extracts containing the barley endosperm LS (bepl10) were devoid of AGPase activity (29), bacterial cell extracts expressing the potato (2), or maize endosperm (30) LS displayed AGPase activity at levels slightly higher or comparable to cell extracts containing expressed SS suggesting that the LS possesses catalytic activity. However, in all cases no further efforts were undertaken to purify and enzymatically characterize the small amounts of soluble LSs present in these crude extracts.

Because the heterotetrameric LS enzyme form was not available until now, the role of the LS has been indirectly inferred based on the properties of the heterotetrameric enzyme containing SS variants (6, 18, 19, 21, 31, 32). Mutations in conserved residues putatively required for Glc 1-P binding or for catalysis in the SS but not in the LS drastically lowered enzyme activity (18), suggesting that SS was solely responsible for catalysis of the heterotetrameric enzyme. This view was also supported by the restoration of catalysis of the heterotetrameric enzyme containing the catalytically silenced D143N SS mutant ($SS^D$) by the introduction of K41R and/or T51K substitutions in the LS (7). Although these results assume that the observed catalytic activity of this mutant AGPase is generated exclusively by the LS, direct insights on its role in enzyme function are more likely to be gained by isolation and enzymatic characterization of a homotetrameric LS.

In this study, we demonstrate that a single mutation, S302N, significantly increases the solubility of the LS and, thereby, facilitating its self-assembly to form a quaternary structure. This condition enabled us to study the enzymatic properties of the $L^N$ homotetramer and compare it to the SS homotetramer and LS-SS heterotetrameric forms. The $L^N$ homotetramer is catalytically very inefficient as its enzyme activity (0.4 unit/g) is just above background controls under our assay conditions (Table 1). The extent of $L^N$ activity is comparable to the catalytic silenced $SS^D$ homotetramer (0.3 unit/g). When assembled together, the resulting $L^NS$ exhibits enzyme activity (0.4 unit/g) in the absence of 3-PGA comparable to the $L^N$ and $SS^D$ homotetrameric forms. In the presence of 3-PGA, however, the
L\textsuperscript{N} responds differently depending on whether it is in the homotetrameric or heterotetrameric form with S\textsuperscript{S}. The L\textsuperscript{N} (as well as the L\textsuperscript{RKN}) homotetramer does not respond to 3-PGA but, when assembled with S\textsuperscript{S}, shows a 30-fold increase in enzyme activity. This enhancement is more readily evident when one compares the activities of L\textsuperscript{RKN} and S\textsuperscript{S} homotetramers to the heterotetrameric enzyme composed of these subunit types. Enzyme activity of L\textsuperscript{RKN}S\textsuperscript{S} is 3400 units/g, whereas those for L\textsuperscript{RKN} and S\textsuperscript{S} homotetramers are 320 units/g and 0.3 units/g, respectively. The L\textsuperscript{RKN}S\textsuperscript{S} enzyme displays a specific activity 11-fold greater than that individually contributed by L\textsuperscript{RKN} and S\textsuperscript{S} homotetramers (Table 1). Hence, the catalytic potential of L\textsuperscript{RKN} is suppressed in the homotetrameric form and/or is activated in the heterotetramer. This behavior is much different from that exhibited by the S\textsuperscript{W} homotetramer where only the allosteric regulatory properties are affected by the absence of the LS. This enhancement of the catalytic rate by the L\textsuperscript{N} and L\textsuperscript{RKN} subunits when assembled with catalytically silenced S\textsuperscript{S} indicates that the LS-3-PGA binding sites are functional when operating in conjunction with the SS but not in the homotetrameric form (Table 1 and Fig. 2). Overall, these results show that the LS possesses catalytic activity, albeit much lower than the SS. Moreover, the increased responsiveness to 3-PGA activation by the heterotetramers (L\textsuperscript{N}S\textsuperscript{S} and L\textsuperscript{RKN}S\textsuperscript{S}) compared with homotetramers (L\textsuperscript{N}, L\textsuperscript{RKN}, and S\textsuperscript{S}) re-confirms our earlier conclusion that the enzymatic properties of AGPase are a product of synergism between LS and SS interactions (6, 9).

Wild-type AGPase heterotetramer shows hyperbolic activation by 3-PGA (Fig. 2A). The S\textsuperscript{W} homotetramer also displays a typical hyperbolic activation curve in response to elevating 3-PGA concentrations but its A\textsubscript{0.5} value (10\textsuperscript{4} \textmu M) is >130-fold larger than the wild-type enzyme (Fig. 2, D versus A, and Table 1). Interestingly, the LS homotetramers (L\textsuperscript{RKN} and L\textsuperscript{N}) were unresponsive up to 1 mM 3-PGA, and L\textsuperscript{RKN} showed a slight inhibition in activity at higher 3-PGA concentrations (Fig. 2, E and F). This lack of 3-PGA activation by L\textsuperscript{RKN} is somewhat surprising, because the activator mimic, pyridoxal phosphate, specifically labels two lysine residues at positions 415 and 453 (25), which are conserved in the SS. Moreover, site-directed mutagenesis of these lysine residues in the LS lowered the sensitivity of the heterotetrameric enzyme (3- to 12-fold) to 3-PGA activation (31). Although similar substitutions in these conserved Lys residues in the SS resulted in a much greater (54- to 3090-fold) inactivation, these results support the involvement of the LS in determining the net allosteric regulatory properties of the heterotetrameric enzyme. This view is best supported by the regulatory properties of the L\textsuperscript{N}S\textsuperscript{S}, L\textsuperscript{RKN}S\textsuperscript{S}, and L\textsuperscript{RKN}S\textsuperscript{S} enzymes whose catalytic activities are contributed nearly solely by the LS in the presence of 3-PGA (Table 1), because the S\textsuperscript{S} lacks the essential Asp for binding of the catalytic metal ion. These heterotetrameric enzymes show a 30-fold or more increase in catalytic activity in the presence of 3-PGA.

In addition to its very low catalytic turnover properties, the L\textsuperscript{RKN} homotetramer possesses poor substrate affinities compared with the wild-type SS homotetramer: ~10-fold lower affinities for ATP and 12-fold lower for Glc 1-P irrespectively of whether or not 3-PGA is present. This reduction in ATP affinity by the L\textsuperscript{RKN} homotetramer is also evident when probed with the 8-\textsuperscript{N}\textsubscript{3}-ATP, which exhibits a labeling constant ~3- to 4-fold higher than that seen for the L\textsuperscript{RKN}S\textsuperscript{W} or S\textsuperscript{W} heterotetramer. This less efficient labeling of LS in the homotetrameric form as compared with more efficient labeling in the heterotetrameric form (6) indicates that ATP binding sites in the LS homotetramer form have an altered conformation than when the LS is assembled with the SS. Nevertheless, the efficient labeling of the LS with 8-\textsuperscript{N}\textsubscript{3}-ATP (Fig. 4) and the ability to elevate the enzyme activity of the heterotetrameric enzyme containing the catalytically silenced SS or homotetrameric LS by the introduction of K41R and T51K substitutions indicate that the substrate binding sites of the LS have been preserved, but the catalytically capacity of this subunit has been compromised (7).

Kinetic analysis revealed that the L\textsuperscript{RKN}S\textsuperscript{S} heterotetramer mutant shows a 4-fold increase in 3-PGA sensitivity over that of L\textsuperscript{RKS}S\textsuperscript{S} (Table 1) with only a marginal change in catalytic rate (Table 2). This enhanced up-regulatory properties gained by the S302N mutation is likely the primary basis responsible for the elevated glycogen production by bacterial cells. Interestingly, Asn at position 302 is highly conserved in nearly all other wild-type AGPase sequences: the only exceptions are two tomato LSs (GenBank\textsuperscript{TM} accession nos. U88089 and U81033). To date, three LS forms have been identified from potato (33). It should be noted that the other two LS forms (GenBank\textsuperscript{TM} accession nos. X76136 and X74982) possess an Asn residue at position 302, implicating analogous function with the L\textsuperscript{N} mutant. Our modeled LS structure (Fig. 6) shows that substitution of Ser\textsuperscript{302} to Asn generates a new hydrogen bond between...
the side chain of Asn\textsuperscript{302} and backbone of Gly\textsuperscript{57} in the glycine-rich loop, which is closely associated with the regulation and catalysis of the enzyme (6, 7, 9, 21, 32). Thus, this change in the loop structure is likely responsible for the up-regulatory properties of the AGPase heterotetramer. Moreover, this conformational change also increases the solubility of the subunit most likely by facilitating folding of the polypeptide to a mature soluble state. However, S302N replacement does not significantly affect the redox status of the enzyme (Fig. 7).

In conclusion, isolation and characterization of the LS homotetramer of the potato tuber AGPase enabled us to demonstrate that the potato tuber LS possesses catalytic and regulatory properties, which are distinct from the SS homotetramer. These properties differ heavily depending on its quaternary form. Other than functional substrate binding and possibly effector binding sites, the LS homotetramer is essentially devoid of allosteric regulatory properties. However, the catalytic potential of LS is partially unmasked, and responses to regulatory change also increase the solubility of the subunit most likely by facilitating folding of the polypeptide to a mature soluble state. However, S302N replacement does not significantly affect the redox status of the enzyme (Fig. 7).

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Direct Appraisal of the Potato Tuber ADP-glucose Pyrophosphorylase Large Subunit in Enzyme Function by Study of a Novel Mutant Form
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doi: 10.1074/jbc.M707447200 originally published online January 16, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M707447200

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