Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 464–477
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Catalytic implications of the higher plant ADP-glucose pyrophosphorylase large subunit

Seon-Kap Hwang [a], Shigeki Hamada [a,b], Thomas W. Okita [a,*]

[a] *Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA*
[b] *Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

Received 27 July 2006; received in revised form 23 October 2006
Available online 4 January 2007

Abstract

ADP-glucose pyrophosphorylase, a key regulatory enzyme of starch biosynthesis, is composed of a pair of catalytic small subunits (SSs) and a pair of catalytically disabled large subunits (LSs). The N-terminal region of the LS has been known to be essential for the allosteric regulatory properties of the heterotetrameric enzyme. To gain further insight on the role of this region and the LS itself in enzyme function, the six proline residues found in the N-terminal region of the potato tuber AGPase were subjected to scanning mutagenesis. The wildtype and various mutant heterotetramers were expressed using our newly developed host-vector system, purified, and their kinetic parameters assessed. While $P_{17}L$, $P_{26}L$, and $P_{55}L$ mutations only moderately affected the kinetic properties, $P_{52}L$ and $P_{66}L$ gave rise to significant and contrasting changes in allosteric properties: $P_{66}L$ enzyme displayed up-regulatory properties toward 3-PGA while the $P_{52}L$ enzyme had down-regulatory properties. Unlike the other mutants, however, various mutations at P_{44} led to only moderate changes in regulatory properties, but had severely impaired catalytic rates, apparent substrate affinities, and responsiveness to metabolic effectors, indicating Pro-44 or the LS is essential for optimal catalysis and activation of the AGPase heterotetramer. The catalytic importance of the LS is further supported by photoaffinity labeling studies, which revealed that the LS binds ATP at the same efficiency as the SS. These results indicate that the LS, although considered having no catalytic activity, may mimic many of the catalytic events undertaken by the SS and, thereby, influences net catalysis of the heterotetrameric enzyme.

Keywords: Potato tuber AGPase; ADP-glucose pyrophosphorylase; Large subunit; Allosteric regulation; Catalytic roles; *In vitro* mutagenesis; Photoaffinity labeling

1. Introduction

ADP-glucose pyrophosphorylase (EC 2.7.7.27; AGPase) catalyzes a pivotal reaction in controlling carbon flux in the α-glucan (starch/glycogen) pathway in plants and bacteria (Preiss, 1984; Preiss et al., 1991; Slattery et al., 2000; Ballicora et al., 2004). While the AGPases from enteric bacteria are activated by fructose-1,6-diphosphate and inhibited by AMP, the enzymes from plants and some photosynthetic algae are activated and inhibited by the major photosynthetic metabolites 3-PGA and inorganic phosphate (Pi), respectively. These regulatory properties are not ubiquitous as the major seed enzyme activities from wheat (Gomez-Casati and Iglesias, 2002) and barley (Kleczkowski et al., 1993a,b; Doan et al., 1999) are relatively unresponsive to these effectors.

Despite possessing similar catalytic and allosteric regulatory properties, the bacterial and higher plant enzymes have different structures. AGPases from bacteria and cyanobacteria are homotetrameric in composition consisting of a single subunit type (α_4), whereas the enzymes from plants have a $\alpha_2\beta_2$ structure comprised of a pair of large subunits (LSs) and a pair of small subunits (SSs) (Iglesias et al., 1993). The subunit types appeared to play different roles in enzyme function. In the absence of the LSs, the

* Corresponding author. Tel.: +1 50 933 53391; fax: +1 50 933 57643.
E-mail address: okita@wsu.edu (T.W. Okita).

0031-9422/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.phytochem.2006.11.027

SSs are capable of self-assembly into bacterial-like (S_4) AGPases. While the catalytic activity of the potato tuber S_4 is still regulated by effector molecules, it displays significant reduction in 3-PGA sensitivity and increased sensitivity to Pi inhibition (Ballicora et al., 1995; Salamone et al., 2000). Although possessing a related primary sequence, the potato tuner LSs are incapable of efficient self-assembly into a catalytically active oligomeric enzyme (Iglesias et al., 1993; Doan et al., 1999). The presence of the LSs enhances the allosteric properties of the heterotetrameric enzyme suggesting that the LS is a regulatory subunit. This functional assignment was supported by results from a photoaffinity labeling studies using pyridoxal 5-phosphate, which mimics 3-PGA in activating the enzyme. The potato tuber LS was preferentially labeled at three sites whereas the SS was labeled at a single site (Ball and Preiss, 1994). However, site directed mutagenesis of the putative lysine residues located at or near the 3-PGA activator binding site in the SS markedly reduced sensitivity to 3-PGA compared to corresponding mutations in the LS (Ballicora et al., 1998). This result suggested that the LS was not a regulatory subunit in providing the effector binding sites but, instead, served as a modulator in affecting the allosteric regulatory properties of the catalytic SS (Ball and Preiss, 1994; Ballicora et al., 1995, 1998; Greene et al., 1996a,b; Laughlin et al., 1998; Kavakli et al., 2001). Several point mutations in the SS also drastically enhanced the up-regulatory properties of the heterotetrameric enzyme supporting the dominant role of this subunit (Salamone et al., 2000; Salamone et al., 2002). Results from other studies (Cross et al., 2004; Hwang et al., 2005), however, do not support this narrow functional assignments for the LS and SS. Analysis of maize-potato mosaics of AGPase indicated that both subunit types contributed to regulatory properties (Cross et al., 2004). This view was also supported by the analysis of AGPase enzymes containing various combinations of wildtype and mutant LSs and SSs (Hwang et al., 2005). The net regulatory properties of enzymes containing different combinations of up-regulatory and down-regulatory LSs and SSs were significantly higher than the combined properties of each subunit type, indicating that the two subunits interact synergistically. Thus, both subunits contribute to the allosteric regulatory properties of the heterotetrameric enzyme.

Available evidence indicates that the N-terminal region of the potato tuber LS plays a major role in specifying the enzyme's allosteric regulatory properties. Deletion of the first 17 amino acids of the LS gave rise to marked increases both in sensitivity to 3-PGA and in resistance to Pi inhibition with little change in catalytic properties (Laughlin et al., 1998). Further deletion of 11 additional amino acid residues reversed the effect of the smaller 17 amino acid deletion. The deletion mutant showed roughly unchanged substrate affinities and 3-PGA sensitivity from the wildtype condition. Random mutagenesis studies showed that a single mutation at Glu-38 or Pro-52 in the LS (designated $E38^{LS}$ or $P52^{LS}$) significantly altered the allosteric behaviors of the AGPase. $E_{38}K^{LS}$ resulted in an enzyme with enhanced sensitivity to 3-PGA activation and enhanced resistance to Pi inhibition (up-regulatory phenotype) while $P_{52}L^{LS}$ rendered the enzyme with the opposite down-regulatory phenotype (Greene et al., 1996a,b, 1998; Hwang et al., 2005).

In this study, we employed a leucine-scanning mutagenesis to further investigate the role of the N-terminal region of the potato tuber LS in the enzyme function of AGPase. Pro were selected for mutagenesis as this residue has restricted conformational flexibility and hence its replacement is likely to cause significant changes in structure and, in turn, protein function and stability (Reiersen and Rees, 2001). Structural and kinetic analyses indicate that mutations of three proline residues located between residues 44 and 66 dramatically alter enzyme function, each having distinct effects on allosterism or catalysis. Replacement of $P52^{LS}$ and $P66^{LS}$ produced enzymes with down- and up-regulatory properties, respectively. Interestingly, $P44^{LS}$ is critical for catalytic activity, substrate affinities, and responsiveness to metabolic effectors. We also show that the LS is labeled by 8-azido-ATP, a substrate analog, at a level comparable to SS. This empirical result implicates a more direct involvement of the non-catalytic LS in the formation of ADP-glucose by the heterotetramer AGPase.

2. Results

2.1. New host cell and plasmids for AGPase expression

During the course of our studies on AGPase, efforts were continually directed at improving the expression of the recombinant enzymes. Production of recombinant AGPase has relied on the *glgC*-deficient strain AC70R1-504 (Ballicora et al., 1995) which has been used for the expression of several plant AGPases. Despite the general utility of this host strain, it contains active protease activities which not only can significantly reduce net AGPase activity but could also potentially modify the enzyme's properties (Salamone et al., 2000). These features prompted us to generate a new host strain lacking both protease and endogenous AGPase activities. The *Escherichia coli* strain ER2566 was selected to generate a *glgC* null mutation because this strain lacks the major protease activities (*lon* and *ompT*) as well as the endonuclease A (*endA*), which aids in plasmid stability. The *E. coli* AGPase structural gene *glgC* was inactivated using the pKO-1 recombination system (Link et al., 1997) which inserted a 0.72 kb fragment of bacteriophage λ DNA into the N-terminal coding sequence of the gene to yield strain EA345 (Fig. 1a).

A potential complication in the study of the heterotetrameric AGPase mutants is the contamination from homotetrameric enzyme forms, a serious problem in those cells which preferentially express the SS compared to the LS due to differences in promoter strength. To minimize this