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Research article

Kinetic commitment in the catalysis of glutamine synthesis by GS1 from Arabidopsis using $^{14}N/^{15}N$ and solvent isotope effects

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ABSTRACT

Glutamine synthetase (GS, EC **6.3.1.2**) catalyzes the production of glutamine from glutamate, ammonium and ATP. Although being essential in plants for N assimilation and recycling, kinetic commitments and transition states of the reaction have not been clearly established yet. Here, we examined $^{12}C/^{13}C$, $^{14}N/^{15}N$ and H_2O/D_2O isotope effects in Arabidopsis GS1 catalysis and compared to the prokaryotic (*Escherichia coli*) enzyme. A $^{14}N/^{15}N$ isotope effect ($^{15}V/K \approx 1.015$, with respect to substrate NH_4^+) was observed in the prokaryotic enzyme, indicating that ammonium utilization (deprotonation and/or amidation) was partially rate-limiting. In the plant enzyme, the isotope effect was inverse ($^{15}V/K = 0.965$), suggesting that the reaction intermediate is involved in an amidation-deamidation equilibrium favoring ^{15}N. There was no $^{12}C/^{13}C$ kinetic isotope effect ($^{13}V/K = 1.000$), suggesting that the amidation step of the catalytic cycle involves a transition state with minimal alteration of overall force constants at the C-5 carbon. Surprisingly, the solvent isotope effect was found to be inverse, that is, with a higher turn-over rate in heavy water ($^{D}V \approx 0.5$), showing that restructuration of the active site due to displacement of H_2O by D_2O facilitates the processing of intermediates.

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1. Introduction

Glutamine synthetase (GS, EC **6.3.1.2**) is the key enzyme of nitrogen assimilation and metabolism since it catalyzes the production of glutamine (Gln) from glutamate (Glu), NH_4^+ and ATP (biosynthetic reaction) with divalent cations as follows: $ATP + Glu + NH_4^+ \rightarrow ADP + Pi + Gln + H^+$.

GS is essential for plant growth and development and GS activity has been shown to correlate to grain production and crop yield productivity (Krapp, 2015). Plants have several forms of GS, involved in different metabolic pathways. GS2 is a chloroplastic enzyme that is part of the photorespiratory cycle and N assimilation and forms Gln from Glu which is in turn synthesized by the glutamine oxoglutarate aminotransferase (GOGAT, EC **1.4.1.13**). Plant GS1 is represented by several cytosolic isoforms that are believed to be involved in N remobilization. As such, GS1 activity plays a critical role in N recycling during grain maturation or leaf senescence (for a review, see (Thomsen et al., 2014)). In humans, GS activity contributes to Glu and NH_4^+ recycling thereby preventing Glu neurotoxicity and hyperammonemia, and a decrease in GS activity could be involved in cerebral stroke (Jeitner et al., 2015) or brain malformations (Frieg et al., 2014).

The crystal structure of the enzyme from different organisms has been published, with bound analogues, see e.g. (Liaw and Eisenberg, 1994; Gill and Eisenberg, 2001, 2002; Krajewski et al., 2005; Krajewski et al., 2008). Quite generally, GS forms a multimeric enzyme, with the active site situated at the interface between two subunits. Structural studies have also provided information on the involvement of active site residues. After ordered substrate

Abbreviations: GS, Glutamine synthetase (EC **6.3.1.2**); IRMS, Isotope ratio mass spectrometry; MetSox, Methionine sulfoximide; OPA, Orthophtaldialdehyde.

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binding (ATP, Glu and then ammonium), Glu is phosphorylated to phosphoglutamate (γ-glutamyl phosphate) then ammonium binds and amidation follows (Krishnaswamy et al., 1962; Timmons et al., 1974; Meek and Villafranca, 1980). The active site contains divalent cations (generally two Mg^{2+} ions but under assay conditions, stoichiometry can be changed to three, and the metal can be substituted to Mn^{2+}) which is essential for active site structure and to stabilize phosphate groups of ADP and perhaps, also the phosphoester group in C-5 of phosphoglutamate. Some active site residues also appear to be important for phosphate and Glu carboxylate group stabilization such as Arg 344 and Arg 359, respectively (Eisenberg et al., 2000). The reaction is believed to start with NH_4^+ deprotonation by Asp 50 of the adjacent subunit (residue numbering for the *Escherichia coli* enzyme, see Fig. S1 and Table S1 for residue numbering in the plant enzyme) (Fig. 1). NH_3 liberated within the active site then attacks phosphoglutamate, forming a protonated $CONH_3^+$ amido group, thereby liberating inorganic phosphate. The amido group is then deprotonated and Gln is liberated. Along the reaction, the Glu 327-containing loop plays the role of a flap (so-called Glu-flap) that is believed to isolate the active site from the solvent and prevents intermediates such as phosphoglutamate from escaping and being hydrolyzed. It is also believed that Glu 327 is essential to stabilize the transition state associated with amidation and is the proton acceptor that deprotonates the charged $CONH_3^+$ intermediate to form Gln. The reaction is made possible by the motion of different enzyme domains (Krajewski et al., 2008; Eisenberg et al., 2000; Murray et al., 2013). For instance, enzyme motion triggered by ATP binding facilitates Glu-flap opening to bind Glu and also reorganize the NH_4^+ binding pocket.

Despite the knowledge of potential residues involved in the reaction and chemical events that lead to Gln production from Glu, key questions remain unsolved. First, apart from structural arguments, there is no direct evidence that the last proton acceptor is Glu 327 and other Glu residues closer to the protonated intermediate could be alternative candidates, such as Glu 131. Second, rate-limiting steps have not been well characterized. Gln binding to the enzyme (monitored by pH displacement) can be easily manipulated with the concentration and the nature of the cation (Hunt et al., 1975), showing that the ability of the active site to accommodate proton release upon Gln liberation is very sensitive to chemical conditions and thus, might intervene in the turn-over rate. Accordingly, isotope exchange studies suggest that product liberation (Gln and/or ADP liberation) might be rate-limiting for the catalytic cycle, at least in the prokaryotic enzyme (Wedler and Boyer, 1972). However, important differences have been found between *E. coli* and pea seed (plant GS1 type): the fact that in the pea seed enzyme, NH_3 and Gln can be isotopically (^{15}N) exchanged at moderate Pi (phosphate) concentration (10 μmol L^{-1}) suggests that amidation is potentially reversible (Wedler, 1974). Third, the geometry of the transition state during amidation itself is not well identified. It has been suggested that the reaction proceeds through a tetrahedral transition state and this might be in agreement with the structure of transition state analogues methionine sulfoximine (MetSox, and its phosphate form methionine sulfoximine phosphate) and phosphinotricin and its derivatives (Manderscheid and Wild, 1986; Colanduoni and Villafranca, 1986; Walker et al., 1987; inhibitors reviewed in Eisenberg et al., 2000). Amongst the latter, γ-hydroxyphosphinotricin has a low K_i value (around 1 μmol L^{-1}, even lower than MetSox). That these two inhibitors with a phosphate group (MetSox phosphate and γ-hydroxyphosphinotricin) are the two most efficient GS inhibitors suggests that the transition state associated with amidation is likely not dissociative, that is, with the phosphate group still close to the C-5 atom of the glutamate moiety. In other words, the transition state can be supposed to stay nearly trigonal or alternatively, be symmetrical, with a modest change of the C=O bond order and reciprocal force constants changes between the C atom and phosphate on the one hand, and between the C atom and NH_3 on the other hand (associative transition state).

As an aid in clarifying the kinetic commitments of the catalytic cycle and providing information about the transition state of amidation, we used kinetic isotope effects. Kinetic isotope effects are useful to examine rate-limiting steps because making or breaking a chemical bond fractionate against heavy isotopes ^{13}C and ^{15}N and this fractionation depends on the commitment along the mechanism. Also, solvent isotope effects (replacing H_2O to D_2O) are instrumental in investigating proton-sensitive steps (for a recent example, see (Mauve et al., 2009)). In plant GS2, amidation has been suggested to be partially rate-limiting due to the relatively high $^{14}N/^{15}N$ isotope effect against ^{15}N (about 1.016) during glutamine synthesis and the absence of $^{12}C/^{13}C$ isotope effect (Stoker, 1994), as in the enzyme from *E. coli* (Yoneyama et al., 1993). Isotope effects in the GS1 form are presently unknown. Here, looked at kinetic and solvent isotope effects associated with the GS1-catalyzed biosynthetic reaction (*Arabidopsis* GLN1;2 thereafter denoted as AtGS) and compared with the prokaryotic enzyme (thereafter denoted as EcGS). These two forms exhibit a very different affinity for substrate ammonium. We took advantage of gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS) and liquid chromatography coupled to chemical oxidation and isotope ratio mass spectrometry (LC-co-IRMS) to determine $^{12}C/^{13}C$ and $^{14}N/^{15}N$ isotope effects. Reaction rates and the H_2O/D_2O solvent isotope effect were monitored with either HPLC or ^1H-NMR. Our results show that there is no carbon isotope effect in both species but surprisingly, the reaction fractionates against ^{14}N in the plant enzyme. An inverse solvent isotope effect was found, and D_2O tended to increase the isotope effect against ^{15}N.

2. Material and methods

2.1. Chemicals and enzymes

O-phthaldialdehyde (OPA), ammonium persulfate, orthophosphoric acid, glutamic acid, glutamine, L-glutamic γ-monohydroxic acid and glutamine synthetase from *E. coli* K12 were purchased at Sigma-Aldrich (Saint Quentin Fallavier, France). ^{15}N-labelled ammonium chloride (98% atom) was purchased at Eurisotop (Saint-Aubin, France). The His-tagged plant enzyme GLN1;2 (At1g66200) from *Arabidopsis thaliana* was obtained using the cDNA kindly provided by Prof. Ishiyama in the pQE2 plasmid vector (Ishiyama et al., 2004). After electroporation, *E. coli* DH5α were cultured for one night at 37 °C on LB agar medium with ampicillin (100 mg L^{-1}), and an aliquot was resuspended in 7 mL LB with amipicillin and cultured for one night. DNA was then extracted from a 2 mL sample with the kit PureYield PMS from Promega (Lyon, France). *E. coli* strain BL21 were then transformed by heat shock (42 °C), selected on LB agar medium and cultured in liquid LB with both ampicillin (100 mg L^{-1}) and kanamycin (50 mg L^{-1}). Protein production was induced with IPTG (1 mM) and the culture was kept at 20 °C for one night. After centrigugation, cells were lysed in buffer (50 mM Tris, 0.3 M NaCl, 10% v/v glycerol, 10 mM imidazole, 1 antiproteasic pill, pH 7.5) with a French press (500 bar). After centrifugation (15,000 g, 4 °C), the pellet was discarded. The supernatant was used to purify the enzyme with the resin Ni-NTA HIS-select® (Sigma-Aldrich), with the same buffer at increasing imidazole concentration for eluting. Fractions were dialyzed against Tris-HCl pH 7.5, $MgCl_2$ (10 mM). The amount of proteins was assessed using Bradford assays and the overall purity by PAGE and Coomassie blue

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