Summary of Ph.D. thesis

The allosteric behaviour of chloroplast localized glutamine synthetase of wheat (*Triticum aestivum* L.), operational mechanisms at the level of substrates

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Introduction

Bread wheat is one of our most important cereals for human nutrition. The high yield of this crop can be ensured by optimization of the nitrogen supply or the production of new plant varieties with higher nitrogen use efficiency. For the latter, deep knowledge of metabolic pathways and regulatory factors is indispensable.

The glutamine synthetase (GS2, EC 6.3.1.4) is a multimer enzyme, which uses Mg^{2+} as its cofactor. GS is essential for the normal growth and development of wheat plants, and has a crucial role during the grain filling process, therefore it affects the yield quality and quantity directly. Moreover in the concatenation of the enzymes of the nitrogen uptake pathway GS is the first and only enzyme which is able to bind an inorganic nitrogen form, ammonia, to a carbon skeleton, the glutamic acid, with the concomitant hydrolysis of ATP and the formation of glutamine. Hereby GS connects the carbon and nitrogen metabolic pathways.

There are two isoforms of GS in leaves: the cytoplasmic GS1 and the chloroplast localized GS2. 80% of the total GS activity derives from the GS2 isoform, which plays an essential role in the reassimilation of the photorespiratory ammonia.

Based on the prominent role of GS2 in metabolism and its localization we suggested, that GS2 has allosteric regulation, possibly negative cooperativity. Negative cooperativity means such an allosteric behaviour, which allows a prolonged saturation and a less alternating or throbbing product formation in a wide interval of substrate concentration. This mechanism promotes maintaining the balance between the carbon and nitrogen metabolism at the level of substrates.

We studied the subunit composition of GS by various electrophoretic methods and protein blot. We also investigated the allosteric regulation of GS, and we identified the regulator substrate of the biosynthetic reaction.

GSs have two Mg^{2+} binding sites with different Mg^{2+} affinity. The prokaryotic GSs exhibit allosteric regulation, where the outcome is based on whether Mg^{2+} or Mn^{2+} is bound to the n2 binding site, which has lower Mg^{2+} affinity. The GS of wheat is also affected by metallic ions, the direct binding of Al^{3+} has been investigated. Hence in our studies we examined the effect of two metallic ions, the Al^{3+} and the Mn^{2+}, on the enzymatic mechanisms.

Here are our summarized most important questions:

1) How can be the subunit composition of the native GS isoenzymes characterized? Can we observe the presence of any interaction partners?
2) Does the GS2 enzyme have any allosteric control? Is there an allosteric regulator substrate for GS2? Is there any effector in the measurements that can influence the results of kinetic measurements?
3) What kind of simplified model or mathematical formula can characterize GS2's operation?
4) How the presence of certain metal ions affects the GS-catalysed reaction? What is the effect of Al^{3+} and Mn^{2+} on the dynamics of glutamic acid utilization, how can they affect the activity?

Materials and Methods

The bread wheat variety, *Triticum aestivum* L *Jubilejnaja* 50 was used for the experiments. Plants were grown hydroponically, in 0.5 mM $CaSO_4$ solution for 3 days, than they were transferred to a modified Hoagland solution (Zsoldos et al., 1986). The 1st leaves of 1 week old plants were used for experimental procedures. The plant material was homogenized in 200 mM tris, 1 mM reduced glutathione, 10 % glycerol, 0.1 % protease inhibitor protein extraction buffer (pH 7.5). The extracts were freshly used for further assays. Protein content was determined by Bradford's method (1976).

GS activity was determined on the basis of the modified synthetase reaction. (Pécsváradi et al., 2009; Rhodes et al., 1975)

The proteins were separated by native polyacrylamide gel electrophoresis on a 6.5% gel (Pécsváradi et al., 2009). GS2 was eluted from the gel by physical destruction in protein extraction buffer. The extracts were freshly used for kinetic measurements. The localization of GS isoenzymes was determined by their activity in the native gel (Pécsváradi et al., 2009).

The homogeneity of the isoenzymes was determined by two dimensional gel electrophoretic separations combined with western blotting. The first dimension was native gel described above, than a slice of the native gel was used for separation by 10% SDS gel. Proteins were transferred to PVDF membrane (Nagy et al., 2013). GS isoenzymes were visualized on the membrane by polyclonal GS antibody and alkaline phosphatase conjugated protein A.

SigmaPlot® (Systat Software, San Jose, California) was used to evaluate the resulted kinetic data.

Results

We studied the homogeneity of wheat GS isoenzymes. The wheat GS1 and GS2 isoenzymes are well separable on native gel, which can be seen in our earlier publication (Nagy et al., 2013). The GS subunit composition was further examined by two-dimensional separation (native + SDS Page) with western blot. Both GS1 and GS2 from the leaves gave identical signs in multiple repeated tests (Németh et al., 2018b). The isoenzymes thus have a homogeneous subunit structure and are constructed from specific GS1 or GS2 subunits. This result is in accordance with the literature.

The effect of end products and substrates on total GS activity was investigated in the crude extracts. We showed that either the artificial or the natural product does not cause inhibition of enzyme activity. Thus, these materials do not interfere with product formation and no product inhibition has emerged as a regulatory option (Németh et al., 2018b).

The enzyme has the highest affinity in the crude extract for hydroxylamine and hence the ammonium ion. The high affinity and sudden hyperbolic saturation excluded the possibility that ammonium ion would have a regulatory effect. Besides the ATP saturation curve can be well characterized by Michaelis-Menten kinetics, which also indicates the lack of cooperativity, however, a decrease in activity was observed in the high ATP concentrations after the completion. In case of glutamic acid, the kinetic curve become saturated at a high glutamic acid concentration, and the curve showed 3 hardly observable inflection points. The curve can be well characterized by Hill equation with a Hill coefficient of less than 1, which, together with the presence of the curve's 3 breakpoints, may indicate allosteric negative cooperativity (Németh et al., 2018b).

The results obtained in ATP and glutamic acid substrate assays in the crude extracts were more pronounced in the purified GS2 extracts. The inhibitory effect of ATP becomes indisputable. Based on this result we suggest the existence of an extra ATP binding site, which is not necessary for the stoichiometry of the biosynthetic reaction. This experimental result serves as kinetic evidence for a suggested, undetermined ATP binding site. Moreover based on our results and the literature we suggest that the number of active sites is equivalent with half of the total number of monomers in the complete native oligomer (Németh et al., 2018b).

The previously observed the inflection points of the glutamic acid saturation curves of crude extracts, were concordant with the ones derived from the experiments with purified extracts. The glutamic acid concentrations belonging to the inflection points overlap with the physiological glutamic acid concentration. The inflection points divide the saturation curve to 4 parts, which suggest that GS2 has 4 catalytically active sites. The adequate fitting of Hill equation and the staggered characteristic of the saturation curve suggest the allosteric negative cooperativity of GS2. We suggest that GS2 has an octamer subunit composition, as it has been showed in case of previous non-recombinant plant GS studies, and we exhibit glutamic acid as the allosteric regulatory substrate of the enzymatic mechanism (Németh et al., 2018b).

According to our results the enzymatic operation of GS2 is ascribable by a mathematical function which is a result of the summation of four hill equation with a $n<1$ Hill coefficient, where the consecutively activated subunits - in relation to the increasing glutamic acid concentration - have increasing K_s value (Németh et al., 2018b).

Based on our results of the studies on the metallic ion binding properties of GS we suggest that the substrate-dependent dynamics of

the glutamate saturation is not affected by Mn^{2+} or Al^{3+}. In addition we showed that the binding of Mg^{2+} is essential for GS2 enzyme activity. It is undoubtedly that the dissociation of Mg^{2+} is caused by the presence of Mn^{2+}, which decreases the enzyme activity; however, we could not show the stable, direct binding of Mn^{2+} (Németh et al., 2018a).

In conclusion the operation of chloroplast localized GS2 is driven by allosteric regulation, negative cooperativity, where glutamic acid serves as regulatory substrate. Therefore glutamic acid as an important metabolic intermediate and signal molecule enables the gradual use of the glutamate substrate at the physiological level. Due to negative cooperativity GS is not only a key enzyme of the nitrogen assimilation, but it also has an important role in maintaining of carbon-nitrogen balance, which should be considered during the breeding of GS overexpressing plant varieties.

The most important new scientific results presented in the thesis are as follows:

1) The GS2 isoenzyme of wheat leaves is under allosteric regulation.
2) The background of allosteric regulation is allosteric negative cooperativity driven by the regulatory substrate, which was identified as glutamic acid.
3) To describe this regulation, we have created a new, approximate mathematical model.
4) The mathematical model can be associated with our newly introduced mechanistic model of GS2.
5) Supraoptimal ATP concentration inhibits the activity of GS2 in vitro.
6) In case of wheat GS2 half of the bound Mg^{2+} can be displaced by Mn^{2+}, presumably this change occurs at the n2 metal binding sites, which have lower Mg^{2+} affinity.

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List of publications

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Full papers:

Publications allowing the Ph.D. procedure:

(*Thesis is based on publications marked by *.*)

***Németh, E.**, Nagy Z., Pécsváradi, A.: Chloroplast glutamine synthetase, the key regulator of nitrogen metabolism in wheat, performs its role by fine regulation of enzyme activity via negative cooperativity of its subunits. Frontiers in Plant Science (9):191 **(2018)** DOI: 10.3389/fpls.2018.00191

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IF: 2.724

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Pál, M., Majláth, I., **Németh, E.**, Hamow, K., Á., Szalai, G., Rudnóy, Sz., Balassa, Gy., Janda, T.: The effects of putrescine are partly overlapping with osmotic stress processes in wheat. Plant Science 268: pp. 67-76. **(2018)** DOI: 10.1016/j.plantsci.2017.12.011

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Lectures and other publications:

Hungarian language conference lectures (lecturer):

Németh, E., Bónus, L., Pécsváradi, A.: Binding of manganese to chloroplast glutamine synthetase and its effect on enzyme activity in wheat. Fiatal Biotechnológusok Országos Konferenciája (FIBOK), Budapest, március 28-29., **2018**

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Nyilatkozat

Mint az alább felsorolt tudományos publikációk felelős szerzője hozzájárulok ahhoz, hogy **Németh Edit** a PhD fokozatszerzési eljárásában felhasználja azokat, első szerzőként, ill. társszerzőként.

*__Németh, E.__, Nagy Z., Pécsváradi, A.: Chloroplast glutamine synthetase, the key regulator of nitrogen metabolism in wheat, performs its role by fine regulation of enzyme activity via negative cooperativity of its subunits. Frontiers in Plant Science (9):191 **(2018)** DOI: 10.3389/fpls.2018.00191

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Dr. Pécsváradi Attila