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GLUTAMATE SYNTHASE IN RICE ROOTS. STUDIES ON THE ELECTRON DONOR SPECIFICITY

AKIRA SUZUKI, JEAN-PIERRE JACQUOT and PIERRE GADAL

Laboratoire de Physiologie Végétale Métabolique, ERA CNRS No. 799, Université de Paris-Sud, Centre d'Orsay, Bâtiment 430, 91405 Orsay Cedex, France

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Abstract—Rice root glutamate synthase activity was assayed with various reducing systems. Ferredoxin-dependent glutamate synthase (EC 1.4.7.1) and pyridine nucleotide-dependent glutamate synthase (NADH, EC 1.4.1.14; or NADPH, EC 1.4.1.13) exhibited a strict specificity for the electron donor. The ferredoxin-dependent glutamate synthase from rice roots could accept electrons from photoreduced ferredoxin in an illuminated reconstituted spinach chloroplast system. Thioredoxin, a potent electron carrier, was not able to provide either ferredoxin-dependent or pyridine nucleotide-dependent glutamate synthase with electrons as no glutamate formation was detected in the presence of reduced thioredoxin f or m.

INTRODUCTION

Glutamate synthase in higher plants was first identified in cultured cells [1] in which pyridine nucleotides: NADH (EC 1.4.1.14) or NADPH (EC 1.4.1.13) were found to be the physiological reductants. Ferredoxin-dependent glutamate synthase (EC 1.4.7.1) was detected in green leaves [2], and enzyme activity dependent on reduced ferredoxin was also found in non-photosynthetic young pea plants [3] and pea roots [4]. Both ferredoxin-dependent and NADH-dependent glutamate synthases were characterized in etiolated pea shoots [5], pea cotyledons [6] and algae, such as *Chlamydomonas* [7]. In contrast, reduced ferredoxin was the specific electron carrier for glutamate synthase in halophyte shoots [8] and rice roots [9], and in these tissues the enzyme was inactive with NAD(P)H.

The electron donor specificity of glutamate synthase shows, therefore, a diversity according to the origin of plant tissues. In rice roots, ferredoxin-dependent glutamate synthase is a protein distinct from NAD(P)H-dependent glutamate synthase [10], and the physiological role of ferredoxin-dependent glutamate synthase in non-photosynthetic roots is in doubt since ferredoxin has not been identified in these tissues. Recent studies have shown that ferredoxin-reduced thioredoxin plays one of the principal roles in the electron transport in chloroplastic enzyme reactions [11, 12]. Thioredoxins were also found in non-photosynthetic plant tissues [12–14] in which NADPH is the immediate reductant for these proteins which subsequently transfer electrons in enzyme catalysed reactions [13].

In this study, the system of electron transport to ferredoxin-dependent and NAD(P)H-dependent glutamate synthases in rice roots was examined.

RESULTS

Electron donor specificity

In Table 1, glutamate synthase activities were compared by assaying with various reducing systems as electron donor. Ferredoxin, reduced either with sodium dithionite (DIT) or with chloroplast reconstituted photosystems was the most efficient electron donor. DIT-reduced methyl viologen gave only 17% of the glutamate formation compared to the ferredoxin assay. A similar value of 20% was obtained with NADH or NADPH at a concentration of 0.22 mM. In contrast, NADPH was a slightly preferred electron donor compared to NADH at an increased concentration of 2.2 mM. Glutamate synthase activity assayed with NADPH generated by G6PDH gave *ca* 35% of glutamate formation, being comparable to the value obtained with externally supplied NADPH at 2.2 mM. This indicates the same effectiveness of enzymatically generated NADPH as exogeneously added NADPH for the enzyme reaction. When the NADP glucose-6-phosphate dehydrogenase (G6PDH) system was added to

Table 1. Glutamate synthase activity dependent on various electron donors

Ferredoxin + illuminated chloroplast reconstituted system	100%
Ferredoxin + DIT	101.5%
Ferredoxin + DIT − glutamine	0%
Methyl viologen + DIT	17.3%
NADH 0.22 mM	19.0%
NADH 2.2 mM	28.4%
NADPH 0.22 mM	19.4%
NADPH 2.2 mM	36.5%
NADH 2.2 mM + NADPH 2.2 mM	35.0%
NADP-G6PDH system	35.0%
NADH 2.2 mM + NADP-G6PDH system	34.5%
NADPH 2.2 mM + NADP-G6PDH system	36.0%
Ferredoxin + NADP-G6PDH system	79.3%

The activities are given as values relative to the photochemically reduced ferredoxin-dependent activity which corresponds to 6.57 nmol glutamate formed/min · mg protein.

NADH or NADPH at a concentration of 2.2 mM, no additional glutamate formation was observed, and glutamate synthesis had about the same values as with NADPH alone, supplied either exogenously or generated enzymatically. In contrast, ferredoxin, reduced with the NADP-G6PDH system gave *ca* 80% of the activity compared to DIT or photoreduced ferredoxin assay, which could suggest the transfer of electrons from NADPH to ferredoxin.

Glutamate synthase activity with thioredoxin as electron donor

Thioredoxin, recently found in roots [12, 14] as well as in leaves [15], could be involved in glutamate formation in rice root tissue since thioredoxin can be reduced enzymatically either with ferredoxin or with NADPH followed by the electron transfer in enzyme catalysed reactions derived from the oxidation of thiol. In Table 2, glutamate synthase activities were shown when enzyme was assayed with thioredoxin as an electron donor. Compared to the ferredoxin assay, *ca* 0.8% of the glutamate was formed with chemically (DTT) reduced thioredoxins, showing that neither ferredoxin-dependent nor NAD(P)H-dependent glutamate synthases in rice root tissue accepted electrons directly from reduced thioredoxin. The enzyme activity, with respect to NADPH at a concentration of 2.2 mM, gave *ca* 34% of the glutamate formation, and when NADPH and thioredoxin fraction were added together glutamate formation was decreased to *ca* 50%. In contrast, when NADPH and the thioredoxin fraction were incubated with the standard enzyme preparation, which was previously subjected to gel filtration through a Sephadex G-50 column, a similar glutamate formation was detected with NADPH alone. These results indicate that NADPH was oxidized and electrons were transferred to thioredoxin in the presence of NADP-thioredoxin reductase which was eliminated by gel filtration.

Table 2. Glutamate synthase activities assayed with thioredoxin system

Ferredoxin + DIT	100%
Ferredoxin + DIT − glutamine	0%
Thioredoxin	0.3%
DTT	0%
Thioredoxin + DTT	0.8%
NADPH 2.2 mM	33.5%
Thioredoxin + NADPH 2.2 mM	15.7%
NADPH 2.2 mM*	33.1%
Thioredoxin + NADPH 2.2 mM*	29.8%

Thioredoxins used for the glutamate synthase assay gave the following enzymatic activities when assayed under the standard conditions as in refs. [12, 26]; 2 μg fructose-1,6-bisphosphatase (EC 3.1.3.11) with 10 μg thioredoxin f: 0.02 μmol fructose-1,6-bisphosphate hydrolysed per min, and 1 μg NADP-malate dehydrogenase (EC 1.1.1.82) with 10 μg thioredoxin m: 0.03 μmol NADPH oxidized per min. Glutamate synthase was assayed with 10 μg of thioredoxins f and m added together. The activities are given as relative values to the dithionite reduced ferredoxin-dependent activity which corresponds to 6.34 nmol glutamate formed/min · mg protein.

* Enzyme assay with the standard enzyme preparation which was subjected previously to Sephadex G-50 gel filtration.

Immunoprecipitation analysis

The standard enzyme preparation was subjected to immunoprecipitation (Table 3). When the enzyme activity dependent on chemically reduced ferredoxin was found to be zero in the supernatant fraction, *ca* 20% of the activity compared to the initial ferredoxin assay was observed with NADH or NADPH, the values being comparable to those in the non-immunoprecipitated standard enzyme extract. These results show that reduced ferredoxin and pyridine nucleotides did not serve as the alternative electron donor to pyridine nucleotide-dependent glutamate synthase and ferredoxin-dependent glutamate synthase, respectively.

Table 3. Glutamate synthase activities in the presence or absence of IgG

	− IgG	+ IgG
Ferredoxin + DIT	100%	0%
NADH 0.22 mM	20.0%	18.5%
NADPH 0.22 mM	18.5%	17.5%

The activities are given as relative values to the dithionite reduced ferredoxin-dependent activity in the absence of IgG which corresponds to 6.30 nmol glutamate formed/min · mg protein.

Glutamate synthase activity in chloroplast reconstituted system

Figure 1 shows the time courses of glutamate synthase activities assayed with chloroplast reconstituted system in the light and in the dark. The glutamate formation was dependent on the presence of ferredoxin and thylakoid membrane fraction in the light, and a low activity was found in the dark. The enzyme activity when assayed with increasing concentration of ferredoxin (Fig. 2) and the thylakoid membrane fraction resulted in a concomitant

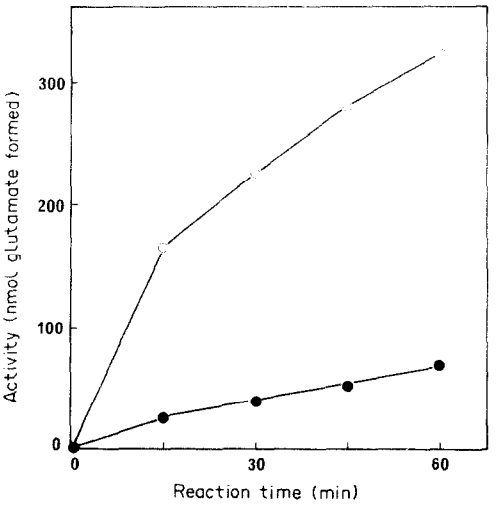

Fig. 1. Glutamate synthase activity in the rice root extract coupled to the reconstituted spinach chloroplasts in the light (○) and in the dark (●).

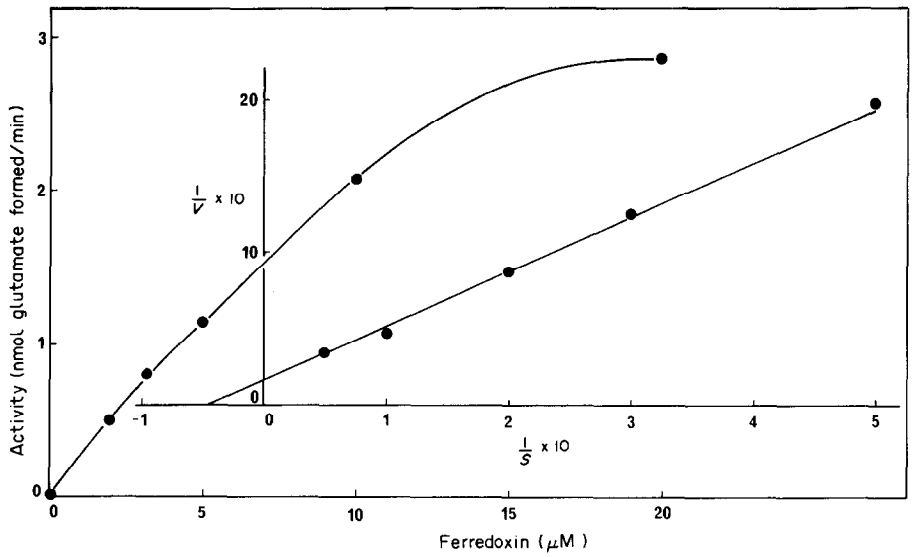

Fig. 2. Glutamate synthase activity with the extract from rice root tissue dependent on different concentrations of photochemically reduced ferredoxin, and corresponding Lineweaver–Burk plots.

glutamate formation, showing that these components in the reconstituted chloroplast electron transport chains could be substituted as the reducing system to ferredoxin-dependent glutamate synthase in rice root tissue. The K_m value of ferredoxin-dependent glutamate synthase in rice root tissue to ferredoxin was estimated to be 20 μM using Lineweaver–Burk plots (Fig. 2). The affinity of ferredoxin-dependent glutamate synthase in rice root tissue to ferredoxin is, therefore, lower than those of the enzymes in green leaf (5.5 μM) and etiolated leaf (4.8 μM) tissues determined under similar assay conditions [16].

DISCUSSION

The results presented here show that glutamate synthase in rice root tissue can utilize electrons from various reductants: reduced ferredoxin, pyridine nucleotides, and artificial dye. In rice root tissue, ferredoxin-dependent glutamate synthase, a distinct protein molecule from NAD(P)H-dependent glutamate synthase [10], does not accept electrons from pyridine nucleotides. Reduced ferredoxin cannot be substituted as an electron carrier to NAD(P)H-dependent glutamate synthase. Glutamate synthase dependent on ferredoxin (EC 1.4.7.1) and pyridine nucleotides: NADH (EC 1.4.1.14) or NADPH (EC 1.4.1.13), therefore, show a strict specificity for the electron donor. It is to be noted that ferredoxin-dependent glutamate synthase operates five-fold faster than NAD(P)H-dependent glutamate synthase *in vitro*. This higher potential activity of ferredoxin-dependent glutamate synthase is found to be supported using the reconstituted chloroplasts in which exogenous ferredoxin is reduced with photosystems in the light. Photoreduced ferredoxin serves as an electron carrier, although the affinity of ferredoxin-dependent glutamate synthase in rice root tissue to ferredoxin is lower than those of ferredoxin-dependent glutamate synthase in rice green leaf and etiolated leaf tissues. In the presence of ferredoxin, glutamate synthase gives a significant amount of glutamate formation which is superior to NADPH-dependent activity when NADPH is generated continuously with G6PDH, one of the characteristic enzymes of the pentose phosphate pathway. The existence of an intermediate which reversibly transfers electrons between NADPH and ferredoxin in rice root tissue could not, therefore, be ruled out. Although NADPH is enzymatically produced here *in vitro*, there is evidence that the pentose phosphate pathway is a potent producer of NADPH required for glutamate formation in roots [17], and the enzymes are in part located in root plastids [18, 19].

Although the immediate source of reductant for ferredoxin-dependent glutamate synthase in rice root tissue is not clear, a direct electron donor, other than NADH or NADPH, should exist to transfer electrons to ferredoxin-dependent glutamate synthase in this tissue. Thioredoxin, recently found in various plant tissues including photosynthetic [14, 15] and non-photosynthetic [12, 14] plant tissues, can be reduced with ferredoxin via ferredoxin-thioredoxin reductase, or it can be reduced with NADPH by virtue of NADPH-thioredoxin reductase in non-photosynthetic tissue [13]. In our experimental conditions, no significant amount of glutamate is formed in the presence of chemically reduced thioredoxin, or thioredoxin which is reduced enzymatically in the extract from rice root tissue, indicating that reduced thioredoxin does not serve as a direct electron carrier to glutamate synthase in rice root tissue in our experimental conditions.

In spite of the glutamate formation by the ferredoxin-dependent glutamate synthase from rice root tissue either with photoreduced ferredoxin or with chemically reduced ferredoxin *in vitro*, the presence of ferredoxin itself or the compounds possibly involved in the transfer of electrons have not been identified in root tissues, nor the mechanism of electron movement in which redox potential is favourable for glutamate formation. In non-photosynthetic tissues, reduced ferredoxin serves for nitrite reduction as an immediate electron carrier [20, 21]. For the nitrite reductase also, electron carrier in non-

photosynthetic tissues is not clarified. Further studies are necessary to elucidate the physiological role of ferredoxin-dependent glutamate synthase in relation to the comparative study on the components in the electron transport system.

EXPERIMENTAL

Plant materials. Seeds of *Oryza sativa* L. cv Delta were incubated in the dark at 29° and grown on Petri dishes containing 0.9% agar made up in 10% Lockard soln [22] containing 10 mM $(NH_4)_2SO_4$ and 10 mM KNO_3 [23]. After 120 hr, the 3 cm root tips were excised as described previously [24]. *Zea mays* caryopses (hybrid, W 64 × W 182 E) were germinated and grown for 14 days on 10% Hoagland soln [25] as described [26].

Standard enzyme extraction. All procedures were carried out at 4°. Root tissues were homogenized with 25 mM buffer (KH_2PO_4–Na_2HPO_4), pH 7.5 containing 14 mM 2-mercaptoethanol and 1 mM EDTA. The debris was filtered through four layers of cheesecloth, and centrifuged at 11 000 *g* for 30 min. $(NH_4)_2SO_4$ was added to the supernatant, and the 30–70% satd $(NH_4)_2SO_4$ fraction was taken. The ppt was dissolved and dialysed for 12 hr against 25 mM NaPi buffer, pH 7.5, containing 14 mM 2-mercaptoethanol.

Glutamate synthase assay. Chemical reduction system. Enzyme activity linked to ferredoxin reduced with DIT was assayed with a standard reaction mixture of 100 μl consisting of 2.25 μmol buffer (KH_2PO_4–Na_2HPO_4), pH 7.3, 0.5 μmol glutamine, 0.5 μmol α-ketoglutarate and enzyme sample. This standard reaction mixture included 2 nmol ferredoxin or 33 nmol methyl viologen which was reduced with 0.9 μmol DIT dissolved in 1.9 μmol $NaHCO_3$. *NADP-G6PDH system.* Enzyme activity linked to NADPH reduced enzymatically with G6PDH (EC 1.1.1.49) was assayed with a standard reaction mixture including 0.1 μmol NADP, 0.1 μmol glucose-6-phosphate, and 0.2 unit of G6PDH. G6PDH was subjected to gel filtration though Sephadex G-50 prior to the enzyme assay in order to eliminate the $(NH_4)_2SO_4$. *DTT-thioredoxin system.* Thioredoxins (f and m) were purified from *Zea mays* leaves essentially following the procedure described in ref. [12]. Enzyme activity was assayed with a standard reaction mixture including 20 μg thioredoxin which was directly reduced with DTT as described in ref. [12]. *Chloroplast reconstituted system.* The standard reaction mixture was added to a chloroplast reconstituted system. It consisted of 1 μmol NaOAc, 2 nmol ferredoxin, 10 nmol 2,6-dichlorophenolindophenol, 1.4 μmol 2-mercaptoethanol, and thylakoid membrane fraction equivalent to 100 μg chlorophyll as described previously [16]. The reaction was carried out with a light intensity of 50 W/m² at the level of the assay tubes at 30°. Chlorophyll contents were determined as in ref. [27]. In all systems, the reaction was stopped by heating at 100° for 1 min, and the assay tubes were centrifuged for 10 min at 10 000 *g*. The enzyme activity was assayed with an aliquot of the supernatant by determining the glutamate formation using a HPLC method [28].

Ferredoxin preparation. Ferredoxin was prepared from spinach leaves by the method of ref. [29]. The concn of ferredoxin was determined as in ref. [30].

Immunoprecipitation analysis. Immunoglobulin G (IgG), raised against ferredoxin-dependent glutamate synthase from rice green leaves, was incubated with the standard enzyme extract. Immunoprecipitated protein was centrifuged and enzyme activity was assayed in the supernatant fraction as described previously [10].

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