Planta (1982) 154:473–476

Planta

The development of NAD(P)H-dependent and ferredoxin-dependent glutamate synthase in greening barley and pea leaves

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Abstract. The activity of NAD(P)H-dependent glutamate synthase (E.C. 1.4.1.14) has been demonstrated in extracts from etiolated shoots of pea (*Pisum sativum* L.) and barley (*Hordeum vulgare* L.). This activity does not significantly alter upon greening of the etiolated shoots, and is at a similar level in light-grown material. Ferredoxin-dependent glutamate synthase (E.C. 1.4.7.1) has low activity in etiolated shoots but increases rapidly on greening. In light grown leaves ferredoxin-dependent activity is 30–40-fold higher than NAD(P)H-dependent activity. It is not considered that the NAD(P)H-dependent glutamate synthase plays an important role in ammonia assimilation in the photosynthetic tissue of higher plants.

Key words: Ferredoxin – Glutamate synthase – Greening (leaves) – *Hordeum* – *Pisum*.

Introduction

Glutamate synthase exists in higher plants in two forms, one utilising NAD(P)H as reductant (E.C. 1.4.7.14) and one utilising reduced ferredoxin (E.C. 1.4.7.1). The ferredoxin-dependent enzyme was originally found in pea chloroplasts (Lea and Miflin 1974; Wallsgrove et al. 1977) and was subsequently shown to occur in both shoot and root tissue from a wide variety of higher plants as well as blue-green bacteria and green algae (Stewart et al. 1980). Although originally isolated from plant tissue cultures (Dougall 1974) NAD(P)H-dependent glutamate synthase has been demonstrated in all non-green tissues in particular roots, root nodules and developing seeds (Stewart et al. 1980). Initially, using relatively insensitive assay techniques, NAD(P)H-dependent activity was not detected in the green leaves of pea, bean, barley and maize nor in cyanobacteria, green algae, mosses and ferns (see Stewart et al. 1980 for review). Recently, use of a much more sensitive assay has led to reports of NAD(P)H-dependent glutamate synthase in pea leaves (Matoh et al. 1980) and in the green alga *Chlamydomonas* (Cullimore and Sims 1981). In this report we present further confirmation of the presence of NAD(P)H glutamate synthase in green leaves.

Materials and methods

Plant material. Peas (*Pisum sativum* cv. Feltham First) and barley (*Hordeum vulgare* cv. Julia) were grown in a controlled environment cabinet as described earlier (Wallsgrove et al. 1979; Wallsgrove et al. 1980). Etiolated plants were grown in a light-proof box at room temperature and hand-dissected barley embryos were grown aseptically as described previously (Bright et al. 1978).

Chemicals. L-[U-^{14}C]Glutamine was obtained from the Radiochemical Centre, Amersham, U.K. and azaserine from Calbiochem-Behring Corporation, C.P. Laboratories, Bishops Stortford, U.K. Purified spinach ferredoxin was a generous gift from Dr. K.K. Rao, Kings College, London, U.K.

Enzyme extraction and assay. Glutamate synthase was extracted by grinding leaves in a pestle and mortar with buffer A (100 mM potassium phosphate pH 7.5, 100 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol) containing 0.1% (v/v) Triton X-100 and 0.5 mM phenylmethyl sulphonyl fluoride at a tissue to volume ratio of 0.25. The homogenate was filtered through "Miracloth", centrifuged at 10,000 *g* for 20 min and the supernatant desalted on Sephadex G.25 equilibrated in buffer A.

Glutamate synthase was assayed in a reaction mixture containing, in a total volume of 100 µl; 4 mM 2-oxoglutarate, 1 mM L-[U-^{14}C]glutamine ($18.9 \cdot 10^4$ Bq mol^{-1}), 10 mM aminooxyacetate, 50 mM potassium phosphate, pH 7.5, and one of the following: 0.6 mM NADH; 0.6 mM NADPH; 10 µg methyl viologen plus 10 µl sodium dithionite solution (25 mg ml^{-1} in 0.3 M $NaHCO_3$); or 45 µM spinach ferredoxin plus 10 µl dithionite solution. The reaction was started by the addition of dithionite or NAD(P)H, incubated at 30° C for 10–15 min and stopped by the addition of 20 µl 15% (w/v) trichloroacetic acid (TCA) containing 2 mM glutamate. After clearing by centrifugation, an aliquot of the supernatant was subjected to high voltage elctrophoresis on Whatmann 3 MM paper in 45 mM Na acetate, pH 5.1, buffer at 3 kV for

0032-0935/82/0154/0473/$01.00

30 min. Areas containing glutamate were cut from the dried paper and the radioactivity measured in a liquid scintillation counter. Chlorophyll was determined by absorption at 654 nm, after extraction of leaf material in cold 95% ethanol, using the extinction coefficients of Wintermans and Demots (1965).

Results and discussion

The original glutamate synthase assay used in this laboratory involved the separation of glutamate from glutamine by paper chromatography and subsequent colorimetric determination of glutamate. This method is limited in its approach in having a relatively low sensitivity for glutamate. Assays of NAD(P)H-dependent glutamate synthase in crude leaf extracts by spectrophotometry are made difficult by the high background rates of NAD(P)H oxidation. Using a modified radiochemical assay and the extraction conditions specified in the Methods, NADH-dependent glutamate synthase activity could be detected in extracts of both pea and barley leaves (Table 1). This activity was dependent on the presence of 2-oxoglutarate and either NADH, or less effectively, NADPH; it was inhibited completely by 1 mM azaserine but not by amino-oxyacetate (up to 10 mM). Varying the extraction conditions did not increase activity; the chosen conditions were based on earlier work which had shown that the optimum extraction conditions for both ferredoxin and NAD(P)H glutamate synthases are very similar. Thiol reagents, EDTA and a protease inhibitor are necessary for maximum enzyme recovery from leaves (Wallsgrove et al. 1977; Tamura et al. 1980) and root nodules (Awonaike et al. 1979; Boland and Benny 1977). Phosphate buffers enhance stability and activity of both forms of the enzyme (Boland and Benny 1977; Wallsgrove et al. 1977) and Triton X-100 is necessary to fully release activity from the chloroplasts (Matoh and Takahashi 1981). The pH optima and kinetic constants of the two enzymes are similar enough to allow a common reaction mixture to be used (Boland and Benny 1977; Wallsgrove et al. 1977). The NAD(P)H-dependent activity differed from that found in *Chlamydomonas* (Cullimore and Sims 1981) in that it was neither cold-labile nor oxygen sensitive, since no increase in extractable activity was found using oxygen-free buffers, and activity was not lost on freezing leaf tissue in liquid nitrogen.

For both plant species NAD(P)H-dependent activity was less than 3% of the ferredoxin-dependent glutamate synthase in light-grown, green, leaves (Table 1). The absolute activity and the ratio of ferredoxin to NADH-dependent activities varied little between experiments, and the two activities were additive, suggesting two independent enzymes as shown in pea leaves (Matoh et al. 1980). Both activities were markedly unstable, the ferredoxin-dependent enzyme being the less stable at 4° C in buffer A. Given this very low activity of the NAD(P)H enzyme, especially in barley, it seemed possible that activity could be due to microbial contamination of the leaf tissue. Excised, sterile barley embryos were grown aseptically in the dark, and extracts made as before. Non-sterile tissue grown under the same conditions was also extracted. The absolute activity of NADH-dependent glutamate synthase and the ratio of ferredoxin to NADH-dependent activities (Table 1) were the same in sterile and non-sterile tissue. We conclude therefore that NAD(P)H activity in barley leaves is not due to bacterial contamination.

Table 1. Glutamate synthase activities in leaf tissue with different reductants

Tissue	Glutamate synthase (nkat g^{-1} FW)			Ratio	
	NADH	Fd	MV	Fd/NADH	Fd/MV
Light-grown pea	1.07	37.4	18.8	35.0	2.0
Etiolated pea	0.98	1.55	0.86	1.6	1.8
Light-grown barley	0.47	18.7	6.9	39.8	2.7
Etiolated barley	0.35	3.44	1.88	9.8	1.8
Etiolated barley (sterile-grown)	0.37	3.66	ND	9.9	–

All values represent the mean of 2–5 experiments
Fd, ferredoxin; MV, methyl viologen; ND, not determined

It can also be seen from Table 1 that assaying glutamate synthase activity with methyl viologen may be a poor measurement of the ferredoxin-dependent activity. Activity with ferredoxin is 2–3-fold higher than with methyl viologen and it is also possible that this latter compound may act as a reductant for the NAD(P)H enzyme (Cullimore and Sims 1981). Hence measurement of methyl viologen-dependent glutamate synthase in plant extracts may be a composite of both activities, and will underestimate the actual activity by a factor of 2 or more.

Whereas little difference was noted between NAD(P)H-dependent glutamate synthase activity in green and etiolated leaves, ferredoxin-dependent activity was 10–20-fold higher in green leaves than in etiolated leaves. Fig. 1 shows the changes in chlorophyll content and in the activity of both glutamate synthases in the leaves on exposing etiolated pea and barley plants to the light. In both species the ferredoxin-dependent glutamate synthase activity more

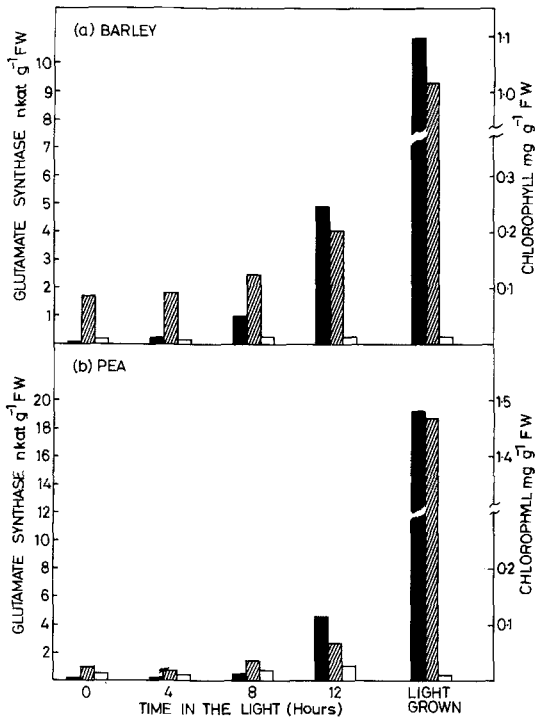

Fig. 1. The development of ferredoxin-dependent and NAD(P)H-dependent glutamate synthase in greening barley and pea leaves. Pea and barley plants, grown in the dark for 7d, were exposed to light (daylight + supplementary tungsten lamps) and samples of leaf tissue harvested at intervals for enzyme and chlorophyll assay. □, NAD(P)H-dependent glutamate synthase; ▨, ferredoxin-dependent glutamate synthase; ■, chlorophyll

than doubled after 12 h, but was still well below the activity in light-grown tissue of the same age. Etiolated pea leaves have only 5% of the ferredoxin-dependent activity of light grown leaves and etiolated barley leaves 18% of the light-grown leaf activity. Slight variations of the NAD(P)H activity were found during greening, but for both species the activity in etiolated leaves was very similar to that in light-grown leaves.

The changes observed are very similar to those reported by Matoh and Takahashi (1981), and it is clear that there is a low, constitutive level of NAD(P)-dependent glutamate synthase, unaffected by light. However, the ferredoxin-dependent activity is very dependent on the greening of the leaf. This situation is analogous to that for the other enzyme in the glutamate synthase cycle, glutamine synthetase. Of the two isoenzymes in green leaves, the chloroplast enzyme glutamate synthase II (GS II) is barely detectable in etiolated barley leaves but comprises more than 60% of the total in light grown tissue (Mann et al. 1980; Wallsgrove et al. 1980). The data in Fig. 1 and Table 1 show that, in green leaf tissue, NAD(P)H-dependent glutamate synthase comprises less than 3% of the total glutamate synthase activity, whereas in etiolated pea and barley leaves it is 33% and 10% respectively. The low level of activity would not have been detected in our original experiments (Lea and Miflin 1974), and is probably insufficient to play a significant role in either primary nitrogen assimilation or in the reassimilation of ammonia released from glycine during photorespiration (Keys et al. 1978). This latter conclusion is supported by data from *Arabidopsis* mutants lacking ferredoxin-dependent glutamate synthase; these mutants are incapable of reassimilating the ammonia released from glycine during photorespiration (Somerville and Ogren 1980), although the activity of NAD(P)H-dependent glutamate synthase in *Arabidopsis* leaves has not yet been examined.

The relative ratio of activities in non-photosynthetic tissue shown in Table 1 agree closely with estimates of the relative contribution of NAD(P)H-dependent glutamate synthase in roots, etiolated shoots and developing cotyledons (Matoh et al. 1979 (a); Matoh and Takahashi 1981; Matoh et al. 1979 (b); and the low relative activity in green leaf tissue has also been shown in maize (Matoh et al. 1979 (a)). *Chlamydomonas* differs from higher plants in that NADH-activity exceeds ferredoxin-dependent activity (Cullimore and Sims 1981). The spatial separation of two glutamate synthase cycles suggested for *Chlamydomonas* would seem not to be true of higher plants, as both forms of glutamate synthase have been shown to be located in the chloroplast (Matoh and Takahashi 1981; Wallsgrove et al. 1979).

We are indebted to Joseph Kueh for his help in growing sterile barley seedlings.

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Received 1 December 1981; accepted 19 January, 1982