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Glutamate Decarboxylase from Barley Embryos and Roots

GENERAL PROPERTIES AND THE OCCURRENCE OF THREE ENZYMIC FORMS

By KOICHI INATOMI* and J. COLIN SLAUGHTER
Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX, U.K.

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Glutamate decarboxylase in extracts of barley embryos has a K_m value for L-glutamate of 22mM and is activated by the addition of pyridoxal phosphate by up to 3.5 times. Sucrose-density-gradient experiments indicate the presence of two enzyme forms with molecular weights 256000 and 120000. The lower-molecular-weight form appears to be relatively inactive and spontaneously associates to the higher-molecular-weight form on storage. The enzyme is inhibited by thiol reagents and the distribution of activity on density gradients is altered in favour of the lower-molecular-weight form by the presence of 2-mercaptoethanol. After removal of the 2-mercaptoethanol spontaneous association to the higher-molecular-weight form occurs. The presence of oxygen in the extraction buffer and in the water during imbibition leads to a relative increase in the higher-molecular-weight form compared with situations where oxygen is excluded. In contrast, glutamate decarboxylase in extracts of 3-day-old barley roots has a K_m value for L-glutamate of 3.1mM and is activated up to 10% by addition of pyridoxal phosphate. The root enzyme occurs as a single species with molecular weight 310000 and this is unaffected by 2-mercaptoethanol although thiol reagents do act as weak inhibitors. The molecular weight is also unaffected by the presence or absence of oxygen in the extraction buffers.

Glutamate decarboxylase (EC 4.1.1.15) is widely distributed in the plant kingdom and although its activity has been associated with senescence by some workers, others have demonstrated a dynamic role for the enzyme during seed germination. The evidence has been discussed previously (Inatomi & Slaughter, 1971) and it seems likely that the enzyme is normally involved in metabolism but the product γ-aminobutyrate, accumulates appreciably in mature tissues only under unfavourable growth conditions such as lack of oxygen. However, Inatomi & Slaughter (1971) showed that in barley embryos during the stage of water imbibition, γ-aminobutyrate accumulated markedly only when oxygen was available. During subsequent aerobic growth the concentration of γ-aminobutyrate steadily declined.

It is possible that the function and nature of glutamate decarboxylase may be different at different stages of development and as previous information on barley glutamate decarboxylase rested on work carried out with enzyme derived from well-developed roots it was decided to investigate the properties of glutamate decarboxylase obtained from barley embryos in order to allow a comparison to be made of the enzymes from the two sources.

* Present address: Suntory Ltd., Yamazaki Distillery, 1023 Yamazaki, Shimamoto-chyo, Mishima-gun, Osaka, Japan

Experimental

Materials

Barley (*Hordeum distichum* var. Zephyr) harvested in 1970 was used for all experiments. Yeast alcohol dehydrogenase was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and bovine liver catalase was obtained from Cambrian Chemicals Ltd., Croydon, Surrey, U.K. L-[U-^{14}C]-Glutamic acid was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. NAD^+ was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., and pyridoxal 5′-phosphate, 2-mercaptoethanol, cysteine, glutathione and dithiothreitol were from Sigma. Scintillation fluid (NE 233) was from Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K. Other chemicals were supplied by BDH, Poole, Dorset, U.K.

Methods

Preparation of embryo extract. Barley was dehusked by soaking in 50% (v/v) H_2SO_4 at room temperature for 2h. The acid was thoroughly washed off with tap water and the residual husks were removed. After another washing with water, the seeds were soaked in 2% (v/v) Chloros solution for 10min to effect sterilization and then washed with sterile water five times. Batches of 300 seeds were soaked in 150ml

portions of sterile water in a 500ml conical flask at room temperature for 18h. The barley was then washed with sterile water, the embryos were severed from the endosperm and again thoroughly washed with sterile water before being homogenized with an ice-cold pestle and mortar in a solution of 0.02M-KH_2PO_4 adjusted to pH6.2 with NaOH, containing 0.032mM-pyridoxal phosphate. The homogenate was subsequently centrifuged at 1000*g* for 10min, and the supernatant used as the enzymic extract.

Preparation of root extract. Seeds (300) of dehusked barley were thoroughly washed and sterilized in 2% (v/v) Chloros solution for 10min and then soaked in running water for 18h. Soaked seeds were placed between wet paper towels in a plastic box and allowed to germinate at 25°C for 3 days. The roots were harvested with the use of scissors and washed with distilled water 5 times. Extraction was carried out as for the embryos except that the buffer strength was increased to 0.05M.

Density-gradient centrifugation. A linear sucrose density gradient was generated from 10ml each of 5 and 20% (w/v) sucrose solution buffered with 0.02M-potassium–sodium phosphate buffer, pH6.2, on a cushion of 50% (w/v) sucrose solution in 22ml polycarbonate centrifuge tubes obtained from MSE (MSE 59209/0173). Pyridoxal phosphate (0.32mM) was included throughout the gradient when it was present in the barley extracts. The experiments with alcohol dehydrogenase and catalase were carried out at pH7.5 in the absence of pyridoxal phosphate. All gradients were used after overnight storage at 0°C.

The sample (1ml) was layered on the density gradient, and centrifugation was carried out in an MSE Superspeed 40 at 30000 rev./min for 22h in a swing-out bucket rotor (3×22ml; MSE catalogue no. 59590). The temperature during the run was maintained between 0° and 1°C.

An ISCO density-gradient fractionator (model 183) was used to separate the gradient into 21 fractions of 1ml each. E_{254} was recorded continuously during fractionation and the sample tubes were stored in an ice bath after collection of the fractions. The fractions obtained were numbered from the less-dense to the more-dense end of the gradient.

Glutamate decarboxylase assay. A technique for measuring the $^{14}CO_2$ liberated from [U-^{14}C]glutamic acid by enzymic action was used. Each assay contained, in a final volume of 0.5ml, 70mM-sodium–potassium phosphate buffer, pH6.2, 0.32mM-pyridoxal phosphate and 30mM-L-[U-^{14}C]glutamate (13.3mCi/mol) in a Universal vial (18m×51mm). A test tube (5mm × 35mm) containing a rolled-glass-fibre disc wetted with 250μl of Hyamine 10-X was placed in the vial. N_2 gas was flushed into the vial from a thin nozzle for 1min, and the vial was then sealed with a Suba Seal stopper. The reaction was initiated by injection of 100μl of enzyme preparation from a micro-syringe, and the vial was incubated at 30°C for 45min after which 0.2ml of 1M-H_2SO_4 was injected to stop the reaction and release CO_2. The sealed vial was allowed to stand overnight at room temperature. The glass-fibre disc was then transferred to a scintillation vial to which 9ml of scintillation fluid (NE 233) was added. After the vial had been left for 1h in the dark, the radioactivity was counted in an ICN Tracerlab Corumatic-200 liquid-scintillation counter. Enzyme activity is expressed as μmol of CO_2 produced/h per ml of enzyme preparation.

Catalase assay. Catalase was assayed spectrophotometrically by the method described in the Sigma Chemical Co. catalogue (1969). Sodium-potassium phosphate buffer solution (0.98ml; 0.1M; pH7.0) containing 13mM-H_2O_2 was placed in a 1ml semimicro-cell which gave an absorbance of 0.550 at 240nm. Enzyme preparation (20μl) was added and the decrease in E_{240} due to the decomposition of H_2O_2 was followed in a Unicam SP 1800 spectrophotometer. Enzyme activity was expressed as μmol of H_2O_2 decomposed/min per ml of the preparation.

Alcohol dehydrogenase assay. Alcohol dehydrogenase was assayed in the following manner (Martin & Ames, 1961). Enzyme preparation (10μl) was added to 0.89ml of Tris–HCl buffer solution (0.05M, pH8.5) containing 3mg of NAD^+, and the reaction was initiated by the addition of 0.1ml of ethanol. The increase in E_{340} at 25°C was recorded in a Unicam SP 1800 spectrophotometer. Enzyme activity is expressed as μmol of NAD^+ reduced/min per ml of preparation.

Results

Kinetic properties of glutamate decarboxylase from barley embryos

The evolution of $^{14}CO_2$ from [U-^{14}C]glutamate under the assay conditions described was linear for up to 60min and directly proportional to enzyme concentration. An apparent K_m value for L-glutamate of 22mM was found. These observations were made in the presence of 0.32mM-pyridoxal phosphate.

Glutamate decarboxylase activity of the crude extract was increased 2.5 to 3.5 times by the addition of pyridoxal phosphate to the assay and a saturation concentration of 0.04mM was found for pyridoxal phosphate. When included in the extraction medium pyridoxal phosphate effectively stabilized the glutamate decarboxylase activity of a preparation stored at 0°C for up to 75h.

Addition of $MgCl_2$, $CaCl_2$ and EDTA to the enzyme preparation at 10mM did not affect glutamate decarboxylase activity. Treatment of the extract with 1% (v/v) Triton X-100 at 0°C for up to 45min had no

effect on enzyme activity but after 85min incubation a 50% loss of activity occurred.

Inhibition of glutamate decarboxylase from barley embryos by thiol compounds

Treatment of extracts of barley embyros with a range of thiol compounds at a concentration of 15mM produce the following inhibition: dithiothreitol, 83%; cysteine, 80%; 2-mercaptoethanol, 59%; GSH, 15%. Inhibition of glutamate decarboxylase by 2-mercaptoethanol was reversible. Treatment of an enzyme preparation with 5mM-2-mercaptoethanol resulted in about a 50% decrease in activity and the removal of the 2-mercaptoethanol by dialysis restored the activity to 95% of the initial activity; the activity in the preparation stored with 2-mercaptoethanol remained low. The results of equivalent experiments where no 2-mercaptoethanol was added confirmed that dialysis itself has no effect on glutamate decarboxylase activity.

Molecular weight of glutamate decarboxylase from barley embryos

Centrifugation of embryo extracts on sucrose density gradients containing 0.32mM-pyridoxal phosphate (Martin & Ames, 1961) gave two peaks of glutamate decarboxylase activity (Fig. 1). The peak in the higher-density region on the gradient was designated enzyme I and that in the lower-density region enzyme II. Assuming that enzyme I and enzyme II both have a partial specific volume of 0.725cm^3/g, $s^0_{20,w}$ values of 11.2 for enzyme I and 7.0 for enzyme II were estimated by comparison with the distances travelled by yeast alcohol dehydrogenase and bovine liver catalase under the same conditions of centrifugation (Cozzani *et al.*, 1970). Estimates of molecular weight, based on the assumption that glutamate decarboxylase is a spherical molecule, were 2.6×10^5 for enzyme I and 1.2×10^5 for enzyme II. For each enzyme, linearity of movement through the gradient was confirmed by centrifuging for different lengths of time up to 60h.

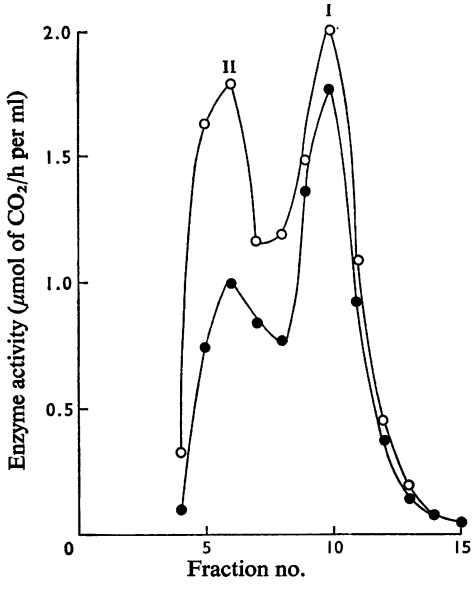

Fig. 1. *Sucrose-density-gradient centrifugation of glutamate decarboxylase from barley embryos*

Barley embryo extract (1ml) was centrifuged on a sucrose density gradient. The glutamate decarboxylase activity of the resultant fractions was assayed either immediately on preparation (●) or after storage at 0°C for 2 days (○).

Relationship between enzyme I and enzyme II

Stability of enzyme I and enzyme II. The stability of enzyme I and enzyme II separated by density-gradient centrifugation was examined by measuring the activity immediately after fractionation and after storage at 0°C. The results (Fig. 1) show that the glutamate decarboxylase activity increased during storage but that the increase was proportionally much greater in enzyme II activity than in enzyme I. The average increase in activity in fractions 4–8 which contain enzyme II was 78% whereas an average increase in fractions 9–13 which contain enzyme I was 21%.

Inhibition of enzyme I and enzyme II by 2-mercaptoethanol. Inhibition of enzyme I and enzyme II by 2-mercaptoethanol was examined by measuring the enzyme activity in the fractions from a sucrose gradient with and without addition of 2-mercaptoethanol to the assay. The results indicated that both enzyme I and enzyme II were inhibited by 2-mercaptoethanol to approximately the same extent.

Effect of 2-mercaptoethanol on the distribution of glutamate decarboxylase on density gradients. The effect of 2-mercaptoethanol on the distribution of glutamate decarboxylase activity was investigated by making a barley embryo extract 5mM to 2-mercaptoethanol before centrifugation on a sucrose gradient. In the case of one tube 5mM-2-mercaptoethanol was included in the gradient and the results are shown in Fig. 2. When the extract was treated with 2-mercaptoethanol and centrifuged on an unsupplemented sucrose gradient, a drastic change in the distribution pattern of the activity occurred. Enzyme I activity was significantly decreased whereas enzyme II activity was very little affected. Inclusion of 2-mercaptoethanol also in the gradient caused more loss of both enzyme I and enzyme II, but again the loss of enzyme I activity was much greater than that of enzyme II.

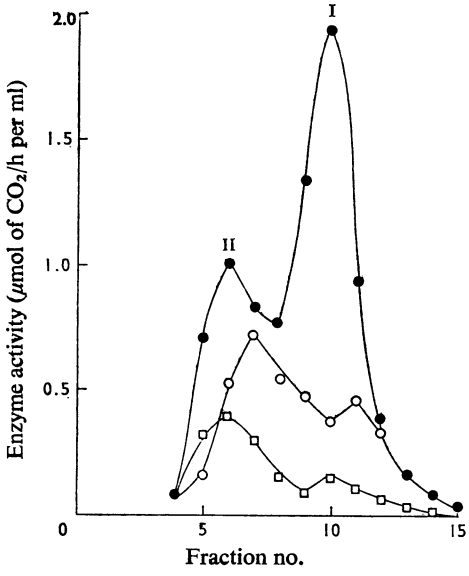

Fig. 2. *Effect of 2-mercaptoethanol on the distribution of barley embryo glutamate decarboxylase on sucrose density gradients*

A barley embryo extract was divided into two portions one of which was made 5mM with respect to 2-mercaptoethanol. After incubation for 1h at 0°C 1ml of each extract was applied to a standard sucrose density gradient and 1ml of the 2-mercaptoethanol-treated extract was applied to a gradient containing 5mM-2-mercaptoethanol. After centrifugation under the standard conditions the glutamate decarboxylase activity of all fractions was assayed immediately after collection. ●, Mercaptoethanol absent from extract and gradient; ○, mercaptoethanol present in extract but not in gradient; □, mercaptoethanol present in both extract and gradient.

Recentrifugation of enzyme I and enzyme II. Samples of enzyme I and enzyme II prepared by sucrose-density-gradient centrifugation of a barley embryo extract were centrifuged again on an identical gradient after 2 days of cold storage (Table 1). The results indicate that after time has been allowed for spontaneous activation, the enzyme II fraction from the original gradient now contains a large proportion of enzyme I. In contrast, the enzyme I fraction from the original gradient appeared to contain almost entirely enzyme I, and only the merest trace of enzyme II activity was observed on recentrifugation. Repetition of this experiment with extracts containing 5mM-2-mercaptoethanol gave essentially the same results (Table 1), but with much lower activities throughout.

Effect of the presence of oxygen in extraction buffers. The effect of extracting barley embryos which had imbibed still water for 18h with buffers saturated with either air or N_2 followed by storage for up to 47h under either air or N_2 was determined (Table 2). When an air-saturated buffer was used

Table 1. *Recentrifugation of enzyme I and enzyme II*

In Expt. 1 an extract of barley embryos obtained from seeds after 18h imbibition of still water was fractionated on a standard density gradient. After storage at 0°C for 2 days the fractions representing the highest activity in the enzyme I and enzyme II zones were dialysed to remove sucrose and then subjected to recentrifugation under the original conditions. The resultant series of fractions were assayed for glutamate decarboxylase activity by using the standard technique except that the specific radioactivity of the [^{14}C]glutamate solution was increased tenfold to cope with the much lower enzyme activities obtained on recentrifugation. The Table shows the activity of the most active fraction in each of the two enzymic zones obtained. Expt. 2 was a repeat of Expt. 1 except that the original embryo extract contained 5mM-2-mercaptoethanol. Enzyme activity is given as μmol of CO_2/h per ml.

		Maximum activity observed on second gradient in:	
Expt.	Location on initial gradient	Enzyme I zone	Enzyme II zone
1	Enzyme I	0.39	0.02
	Enzyme II	0.14	0.10
2	Enzyme I	0.08	0.01
	Enzyme II	0.16	0.08

Table 2. *Effect of the presence of oxygen in extraction buffers on the glutamate decarboxylase activity of barley embryo extracts*

The embryos were removed from barley seeds which had been soaked in still water for 18h at room temperature and divided into two equal portions. An extract was made from each portion in the standard fashion except that in one case the extraction buffer was saturated with air and in the other, saturated with N_2. After preparation, each extract was divided into two portions, one of which was stored at 0°C open to the air and the other was stored at 0°C in a sealed container containing an atmosphere of N_2. AA, Extraction medium saturated with air and preparation stored open to the air; AN, extraction medium saturated with air and preparation stored under N_2; NA, extraction medium saturated with N_2 and preparation stored open to the air; NN, extraction medium saturated with N_2 and preparation stored under N_2. Numbers in parentheses show glutamate decarboxylase activity as a percentage of that found in sample AA.

Time from extraction (h)	Glutamate decarboxylase activity (μmol of CO_2/h per ml)			
	AA	AN	NA	NN
0	8.3	8.3 (100)	4.8 (58)	4.8 (58)
4	9.6	8.4 (88)	7.1 (74)	5.1 (53)
23	8.4	8.3 (99)	7.7 (91)	6.9 (82)
47	7.1	7.1 (100)	6.9 (97)	6.7 (94)

the method of subsequent storage appeared to have little effect on the glutamate decarboxylase activity of the extracts. However, use of an N_2-saturated buffer yielded an extract whose initial glutamate decarboxylase activity was less than 60% of that obtained when an air-saturated buffer was used. For the N_2-saturated extraction buffer, storage under air gave rise to a rapid increase in glutamate decarboxylase activity whereas when the extract was stored under an atmosphere of N_2, a very much slower increase in activity was observed.

Effect of oxygen availability during the uptake of water

The distribution of glutamate decarboxylase activity on standard sucrose gradients was determined for extracts prepared from unsoaked embryos and from embryos obtained after overnight imbibition of still water, aerated water and N_2-flushed water. In all cases extracts were prepared in both air-saturated and N_2-saturated buffers.

The results in Table 3 indicate that glutamate decarboxylase extracted from dry barley embryos with air-saturated buffer behaves mainly as enzyme I. Extraction and centrifugation in N_2-flushed buffers resulted in a decrease in the amount of enzyme I and an increase in activity in the enzyme II zone. When embryos from barley soaked for 18h in still water were used, less activity appeared in the enzyme I zone and more in the enzyme II zone in both aerated and N_2-flushed preparations (Table 3) compared with the results from the dry-embryo experiments. The presence of oxygen during preparation resulted in a relatively higher enzyme I activity and lower enzyme II activity than that in its absence. When seeds were allowed to imbibe fully aerated water, the distribution of the enzyme on sucrose gradients (Table 3) was of a similar type to that in the dry-seed experiment. When air-saturated buffers were used for extraction and centrifugation, most activity appeared in the enzyme I zone. When an N_2-flushed system was used for extraction and centrifugation activity decreased in the enzyme I zone and increased in the enzyme II zone. In the experiment where seeds were soaked under anaerobic conditions, the yield of activity was low under both conditions of extraction, and the distribution of activity on the gradient was very similar for both extracts (Table 3).

Properties of glutamate decarboxylase from barley roots

The properties of glutamate decarboxylase from barley roots were explored by using the techniques applied to the study of the embryo enzyme. Addition of 0.32mM-pyridoxal phosphate to the enzyme assay produced activation of about 10%, and the K_m value for glutamate was estimated as 3.1mM under the standard assay conditions. The enzyme gave a single symmetrical peak on gradient centrifugation. The calculated value of $s^0_{20,w}$ was 12.8 which is equivalent to a molecular weight of 310000. After storage of the fractions from a density gradient run at 0°C for 2 days a slight decrease in activity was noticed and no new peak of activity appeared at any point on the gradient. Inclusion of up to 25mM-2-mercaptoethanol in the extract and gradient did not alter the distribution of glutamate decarboxylase activity on the gradient although at 25mM-2-mercaptoethanol was distinctly inhibitory. The glutamate decarboxylase obtained in root extracts was not affected by the use of air-saturated or N_2-saturated extraction buffers.

Discussion

The experiments reported here reveal a relatively complex situation with regard to glutamate decarboxylase in barley. The evidence of molecular weight, K_m value and the effect of 2-mercaptoethanol and oxygen indicate that the glutamate decarboxylase of 3-day-old barley roots is quite different from the glutamate decarboxylase of the embryo. The different

Table 3. *Effect of oxygen availability on the density-gradient-centrifugation behaviour of barley embryo glutamate dehydrogenase*

The conditions of the experiment are described in the text. Glutamate decarboxylase activity is given as μmol of CO_2/h per ml.

Source of embryos	Nature of extraction buffer	Activity of the most active fraction in: Enzyme I zone	Enzyme II zone
Untreated dry seeds	Air-saturated	8.7	0.8
	N_2-saturated	5.2	1.2
Seeds soaked for 18h in still water	Air-saturated	5.0	2.5
	N_2-saturated	3.4	3.1
Seeds soaked for 18h in air-saturated water	Air-saturated	11.7	1.2
	N_2-saturated	7.8	1.8
Seeds soaked for 18h in N_2-saturated water	Air-saturated	3.6	2.8
	N_2-saturated	3.8	2.5

quantitative response to pyridoxal phosphate may be due to differences in structure between the enzymes from the two different sources but it is possible that there may be more coenzyme in root extracts than in embryo extracts. No information on this point is available for barley, but it has been reported that for wheat the pyridoxal phosphate concentration can increase up to eight times during germination (Zavenyagina & Bukin, 1971). Whether the occurrence of separate embryonic and mature forms of glutamate decarboxylase is common in the plant kingdom is not known (as very little information is available), but it does appear from the published data that the K_m values of glutamate decarboxylase in plants fall into two groups: between 3 and 4mM for wheat leaf (Weinberger & Glendining, 1952), field bean (Ambe & Sohomie, 1963) and carrot (Schales & Schales, 1946) 25mM for wheat embryo, which appears to be the only other embryonic tissue investigated (Cheng *et al.*, 1960). The values for glutamate decarboxylase from barley tissue reported in this paper also fall into these two groups.

Examination of glutamate decarboxylase from embryos showed that the enzyme is particularly affected by oxidation and reduction *in vitro*. On reduction the larger species, enzyme I, with a molecular weight of about 256000 gives rise to the smaller species, enzyme II, with a molecular weight of about 120000 and a very much lower specific activity. In the absence of reducing agents but in the presence of oxygen enzyme II spontaneously gives rise to enzyme I. The mechanism involved is unknown but the most likely explanation seems to be that enzyme I contains a disulphide bond which is crucial for the maintenance of the quaternary structure of the enzyme.

An attempt to study the effect of oxygen availability during imbibition on glutamate decarboxylase (Table 3) revealed considerable variations in the total glutamate decarboxylase activity and in the distribution between enzyme I and enzyme II in the various extracts. Although it is possible that the results are due to a balance *in vitro* between oxidation by molecular oxygen and reduction by endogenous reducing agents whose concentration in the extract depends on oxygen availability during imbibition, it seems likely that the relative proportion of enzyme I to enzyme II within the embryos during the first phase of growth responds to the ability of the cells to obtain oxygen. Under normal circumstances of germination the barley embryo is a tissue which switches from a 'resting' metabolism to an 'active' metabolism on the influx of water and oxygen and it may be that the phenomenon reported here has a function as part of the system by which this switch is achieved.

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