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CONTROL OF GLUTAMATE DEHYDROGENASE FROM *PISUM SATIVUM* ROOTS

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Key Word Index—*Pisum sativum*; Leguminosae; pea roots; glutamate dehydrogenase; allosteric inhibition; zinc, calcium activation.

Abstract—Glutamate dehydrogenase (GDH) was found in soluble and particulate (mitochondrial) fractions of pea roots. The activity of NADH-dependent GDH in fresh mitochondrial extract was increased about 10-fold by addition of zinc, manganese or calcium, but high concentrations of zinc were inhibitory. During storage, GDH activity of the mitochondrial extract slowly increased. The NADH activity was inhibited by citrate and other chelating agents. NADH-dependent reductive amination was also inhibited by glutamate, the product of the reaction; by contrast NADPH dependent activity was relatively unaffected by zinc, chelating agents or glutamate. Sensitivity (of NADH–GDH) to glutamate was lost on purification, but was restored when the enzyme was immobilized by binding to an insoluble support (AE cellulose). Glutamate appears to change the affinity of the enzyme for 2-oxoglutarate.

INTRODUCTION

GLUTAMATE dehydrogenase (GDH) is a key enzyme in the assimilation of inorganic nitrogen by plants. In animal tissues the enzyme appears to have a predominantly dissimilatory role, and there has been extensive work on its regulation, particularly for the preparation from bovine liver. The animal enzyme which is active with both NAD and NADP, may exist in various states of aggregation, and *in vitro* activity is controlled by a number of nucleotides.[1] In micro-organisms there is evidence that there are separate NAD- and NADP-dependent glutamate dehydrogenases,[2,3] and these have complex regulatory properties.[4–6]

Plant glutamate dehydrogenase has been taken to various stages of purification from corn,[7] peas[8] and pumpkin,[9] but no clear indication of regulation was found. We reported extensive purification of the enzyme from pea roots,[10] which appeared to be susceptible to regulation. Indications of regulatory properties have also been shown in studies by King and Wu[11] and Pahlich,[12] but these reports do not outline a regulatory scheme which would be of physiological significance.

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[12] PAHLICH, E. (1971) *Planta* **100**, 222.

Work in this laboratory has indicated that the enzyme could show variability in activity with storage and in NAD^+/NADH activity ratio.[10] Changes in activity which initially appeared to be due to substrate induction[13] were later shown to be due to changes in sensitivity to a chelating agent used in the extraction medium.[14] The present work indicates that crude, freshly extracted mitochondrial NADH-dependent GDH is inhibited by its product, glutamate, and is stimulated by zinc ions. On purification, the enzyme loses much of its ability to undergo changes of activity.

TABLE 1. DISTRIBUTION OF NADH–GDH IN CRUDE FRACTIONS OF PEA ROOTS

	mUnits/g Fresh Root		mUnits/mg Protein	
	Untreated	+ 0·3 mM Zinc	Untreated	+ 0·3 mM Zinc
Total extract	674	997	277	410
Supernatant fraction	330	392	165	197
Mitochondrial fraction	56	594	373	3960
Sum of supernatant + mitochondrial	386	986		

RESULTS

Recovery of Activity in Crude Fractions

Table 1 shows the GDH activity of total extracts from pea roots and the distribution of activity when supernatant (soluble) and mitochondrial (particulate) fractions were separated. Untreated fresh extracts showed little activity in the mitochondrial fraction, and the sum of activities recovered was low compared to activity of the original total homogenate. Addition of Zn^{2+} caused increases in the activity of all fractions, especially in the mitochondrial fraction where a 10-fold stimulation was noted. In the presence of Zn^{2+}, activity recovered in supernatant and mitochondrial fractions was almost exactly equal to activity in the total homogenate. Approximately 60 per cent of this activity was present in the mitochondrial preparation. On a protein basis, the specific activity of GDH from mitochondrial was 9–10 times greater than that of the total homogenate.

TABLE 2. CHANGES IN NADH–GDH ACTIVITY OF MITOCHONDRIAL FRACTION DURING STORAGE

	Storage temp.			
	4°		−15°	
Initial (day 0)	Untreated (9%)	+ 0·3 mM Zinc (100%)	Untreated (9%)	+ 0·3 mM Zinc (100%)
1 day	25	100	—	—
2 days	60	98	—	—
5 days	64	67	12	100
8 days	49	—	20	100
14 days	—	—	41	97
34 days	—	—	64	89

Activities expressed as a percentage, maximum initial activity for this preparation, in presence of zinc, taken as 100%.

[13] JOY, K. W. (1969) *Plant Physiol.* **44,** 849.
[14] JOY, K. W. (1971) *Plant Physiol.* **47,** 445.

Storage Effects

It was noted that assays of mitochondrial activity often showed variations following storage of the extract. Samples were therefore stored at 4° and −15° and assayed at intervals. Table 2 shows that after 2 days at 4°, activity in untreated extracts had increased while Zn^{2+}-stimulated activity remained constant. After 5 days untreated and plus Zn^{2+} activity were almost equal but the activity began to decrease. A similar sequence took place during storage at −15°, though at a much slower rate. Storage also altered the kinetic properties of the enzyme; fresh mitochondrial enzyme showed evidence of substrate inhibition with 2-oxoglutarate (Fig. 1a). Slight inhibition was found with 67 mM ammonium (Fig. 2c), compared with 33 mM (Fig. 2b), for untreated enzyme, stored at −15° for 2 weeks. However, both types of substrate inhibition were substantially lost on addition of zinc. With storage the response of untreated enzyme to 2-oxoglutarate approached that of the zinc-stimulated system (Fig. 1c).

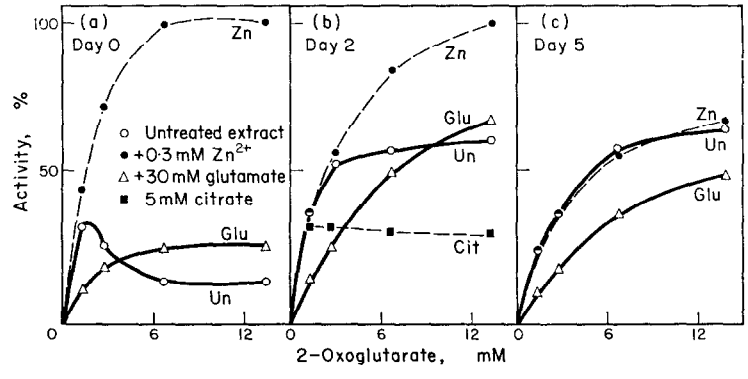

FIG. 1. EFFECT OF AGING AND VARIOUS COMPOUNDS ON NADH-GDH ACTIVITY OF MITOCHONDRIAL EXTRACT.

Mitochondrial extract was assayed with various concentrations of 2-oxoglutarate on day of extraction (a), after 2 days, (b), and 5 days (c) of storage at 4°. Activity is shown for untreated extract or with addition of 0·3 mM Zn^{2+}, 30 mM glutamate or 5 mM citrate. Activity expressed as percentage of maximum activity for this preparation.

Effect of Metal Ions

Freshly extracted mitochondrial enzyme was strongly stimulated by a number of metal ions, as shown in Table 3. Zinc was most effective, with maximum stimulation given by 0·3 mM Zn^{2+} added to the assay. The stimulatory effect was seen only in the presence of higher concentrations of both 2-oxoglutarate and ammonia (Figs. 1 and 2). Concentrations of Zn^{2+} above 0·6 mM caused less stimulation and at 1·5 mM, activity was strongly inhibited. Low concentrations of zinc had little effect on supernatant or purified enzyme, but gave some stimulation of total homogenate, which contained considerable amounts of mitochondrial enzyme (Table 1); high concentrations of zinc caused inhibition of all enzyme preparations.

Manganese was effective at concentrations 10-fold higher than zinc, and also gave decreased stimulation at higher concentrations. Calcium was effective over a wide range of concentration, and showed no indication of an inhibitory effect. Magnesium gave no stimulation of activity, although Mg^{2+} had been shown earlier[10] to overcome EDTA inhibition of the purified enzyme.

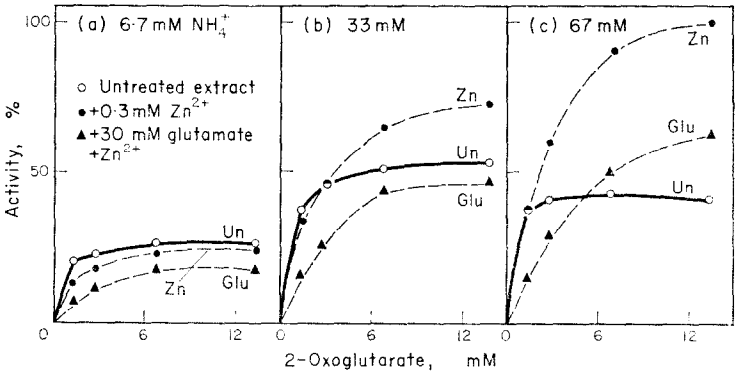

FIG. 2. EFFECT OF AMMONIA CONCENTRATION ON NADH–GDH ACTIVITY OF MITOCHONDRIAL EXTRACT, AND ITS RESPONSE TO VARIOUS COMPOUNDS.
Extract had been stored 2 weeks at −15°. Assays contained ammonium sulphate at concentrations of 6·7 mM (a) 33 mM (b) or 67 mM (c). Activity shown for untreated extract or with addition of 0·3 mM Zn^{2+} or 30 mM glutamate plus Zn^{2+}. Activity expressed as percentage of maximum activity of this preparation.

TABLE 3. EFFECT OF VARIOUS IONS ON NADH–GDH ACTIVITY OF MITOCHONDRIAL FRACTION

Final concentration in assay (mM)	Zinc	Manganese	Calcium	Magnesium
0·15	55%	—	—	—
0·3	100	28	—	12
0·66	—	50	88	11
0·75	97	—	—	—
1·5	8	—	—	—
1·67	—	73	95	12
3·3	—	98	95	13
6·7	—	92	95	—
10·0	—	70	—	—

Activities expressed as percentages. Maximum activity for this preparation set at 100 (with 0·3 mM Zn). On this basis the untreated enzyme had an activity of 12%. Calcium supplied as chloride, other salts as sulphate.

Effect of Citrate, Other Metabolites and Chelators

In an attempt to identify an inhibitor responsible for the low initial activity of mitochondrial GDH, several possible mitochondrial components were tested. Chelating agents capable of binding zinc were also tested, and the results are shown in Table 4. Citrate had a strong inhibitory effect on all crude enzyme preparations—aged mitochondrial, supernatant and total, giving 65–70% inhibition at 5·4 mM. The effect was less pronounced with partially purified enzyme. Malate and succinate caused a small stimulation of mitochondrial activity. Several chelating agents gave varying amounts of inhibition, with EDTA being the most effective. The effect of citrate on response to 2-oxoglutarate is shown in Fig. 1b.

TABLE 4. EFFECT OF SOME ORGANIC ACIDS AND CHELATING AGENTS ON NADH–GDH ACTIVITY

Enzyme Effector (Conc. mM)	Partly purified	Aged mitochondrial	Total homogenate	Supernatant
(Untreated activity)	(100%)	(100%)	(100%)	(100%)
+ Zinc (0·3)	*102*	*129*	*148*	*119*
+ Citrate (2·7)	76	61	63	73
(5·4)	71	37	34	34
+ Malate (2·7)	—	*116*		
(5·4)		*129*		
+ Succinate (2·7)	—	*114*		
(5·4)		*119*		
+ Oxalate (2·7)	95	95		
EDTA (0·02)	—	30		
(0·1)	23	17		
1,10-phenanthroline (0·5)	97	—		
(1·0)	89	100		
8-Hydroxyquinoline-5-sulphonic acid (0·5)	68	45		
(1·0)	14	15		

Activities expressed as percentage of activity of each untreated enzyme. Italicized figures show stimulation Partly purified enzyme had undergone pH precipitation, ammonium sulphate precipitation and dialysis.

Effect of Glutamate and Other Amino Acids

With purified enzyme, addition of glutamate to the assay system for reductive amination caused a small (5–8%) inhibition. With stored crude enzyme preparations, glutamate produced a somewhat greater inhibition (of maximum potential activity) and the apparent K_m for 2-oxoglutarate was increased by about 3-fold to approx. 12 mM, as determined by double reciprocal plot. The apparent K_m for ammonia was relatively unaffected. For mitochondrial enzyme, the activity with glutamate was similar regardless of whether or not Zn^{2+} was present in the assay (compare Fig. 1, only glutamate added, with Fig. 2, glutamate added with Zn^{2+}). Somewhat unexpectedly, glutamate in some cases actually stimulated 'untreated' activity of crude extracts and overcame the substrate inhibition (Figs. 1a, 2c, 3). The difference in response to a range of concentrations is shown in Fig. 3. In the presence of Zn^{2+} very low concentrations of glutamate gave a slight stimulation of activity, with progressively increasing inhibition at higher concentrations. Several other amino acids were tested at concentrations of 30 mM. D-Glutamate gave an even stronger inhibition of maximal activity than did L-glutamate, but D-glutamate could not reverse the substrate inhibition of the untreated fresh mitochondrial enzyme and did not cause any apparent stimulation of activity of this preparation. Glutamine, alanine, aspartate and proline had little effect on enzyme activity. Arginine gave some inhibition of enzyme activity, but this was reversed by addition of Zn^{2+} or Ca^{2+}, and thus appeared to be due to a chelating effect.

Relation of NADH-, NADPH- and NAD^+-Dependent Activity

Although NADH-dependent assimilatory activity is usually the greatest exhibited by the enzyme, activity can also be measured with NADPH and deaminating activity with NAD^+ (Table 5). Activity with NADPH and NAD^+ could be assayed in mitochondrial extracts, but Zn^{2+} caused no stimulation of these activities on contrast to the large change caused in

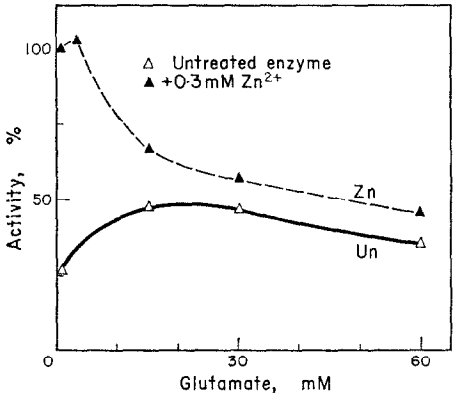

FIG. 3. EFFECT OF GLUTAMATE ON NADH–GDH ACTIVITY OF MITOCHONDRIAL EXTRACT.

Extract had been stored 10 days at −15°. Activity is shown for untreated enzyme or with addition of 0·3 mM Zn^{2+}. Activity expressed as percentage of maximum activity of this preparation with Zn^{2+} alone.

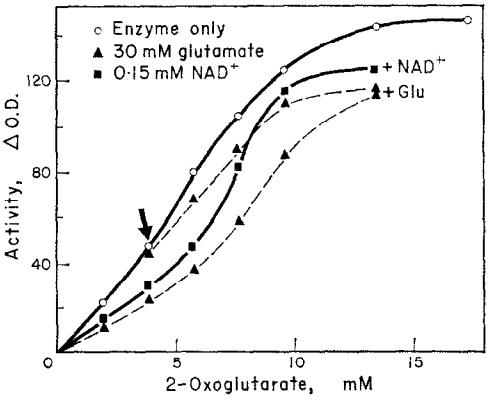

FIG. 4. NADH-DEPENDENT ACTIVITY OF IMMOBILIZED GDH PREPARATION, AND INFLUENCE OF VARIOUS COMPOUNDS.

Activity assayed with successive additions of 2-oxoglutarate with immobilized enzyme only and in presence of 30 mM glutamate or 0·15 mM $NAD.^+$ In one run glutamate was added where indicated during the experiment. The column of GDH preparation was 3·4 × 1 cm dia. Flow rate 13 ml/min. Activity recorded as $\Delta E340$/ min.

NADH activity. NADPH activity showed little response to glutamate. Zinc stimulation (of NADH dependent activity) was not observed in phosphate buffer, but a slight cloudiness on addition of zinc probably indicated precipitation, and thus inavailability, of zinc.

TABLE 5. COMPARISON OF DIFFERENT GDH ACTIVITIES OF MITOCHONDRIAL ENZYME

buffer Treatment	NADH	NADPH	NAD^+
Tris			
Untreated	20%	7	7
+ Zn^{2+} (0·3 mM)	100	6	5
+ Glutamate (30 mM)	44	5	–
Phosphate buffer			
Untreated	23	14	4
+ Zn^{2+} (0·3 mM)	24	14	3
+ Glutamate (30 mM)	49	11	–

Activities expressed as percentage of maximum activity for this preparation, calculated from rates of oxidation of NADH or NADPH with 2-oxoglutarate and ammonia, or NAD^+ reduction with glutamate. PO_4 buffer was 0·1 M, pH 7·75.

Factors Affecting Immobilized Enzyme

Purified GDH was covalently bound by glutaraldehyde linkage to AE cellulose. The immobilized preparation retained activity yet it can be assumed that subsequent changes in state of subunit association of the enzyme would be prevented by the firm binding to the cellulose support material.

Figure 4 shows the effect of increasing 2-oxoglutarate concentration on the activity of immobilized enzyme, and the effect of the presence of glutamate and NAD^+. Points for each curve were obtained from a single run during which 2-oxoglutarate concentration was progressively increased. With 2-oxoglutarate alone the curve was somewhat sigmoid, but addition of glutamate emphasized its sigmoid character and changed the response to 2-oxoglutarate. Addition of glutamate during the run did not immediately change the activity but depressed activity only on subsequent addition of 2-oxoglutarate. The curve also became strongly sigmoidal on addition of NAD^+. EDTA (0·1 mM) and citrate (2·5 mM) had little or no influence on the activity of the immobilized enzyme. D-Glutamate was even more effective than L-glutamate in altering the response to 2-oxoglutarate. Aspartate had little effect, on some occasions producing a slight stimulation of activity.

DISCUSSION

About two-thirds of the glutamate dehydrogenase was recovered from a particulate fraction of pea roots, when appropriate measures were taken to ensure assay of maximum activity. This is in agreement with the observations of several other workers.[11,15,16] The remaining third of the activity may be present in the soluble cytoplasmic fraction *in vivo*, or may be released from mitochondria during extraction. Some of the variability in GDH activity of crude preparations may now be explained in terms of reduced activity of freshly extracted enzyme which is increased rapidly by addition of zinc and some other metal ions, and more slowly by aging of the extract. This is particularly apparent for the mitochondrial enzyme which is stimulated some 8- to 10-fold by zinc ions when freshly extracted; during several days storage at 4° the enzyme gradually attains this maximum activity while the stimulation by zinc declines. Some 'storage activation' is seen in a few hours, which can lead to erratic results if assays are performed over the course of a day. However, reliable measurements of maximum *potential* activity can be obtained in the presence of Zn^{2+} or Ca^{2+}. For routine assays Ca^{2+} may be preferable since it is active over a greater range of concentration than is Zn^{2+}, and does not inhibit at several times the optimal concentration. It should be noted that use of phosphate rather than Tris buffer in the assay system can also give variations in activity and behaviour of the enzyme.

The mechanism of rapid activation by metal ions (further discussed below) and slow storage activation requires explanation. Since it is unlikely that the metal ion concentration in the stored enzyme changes, storage activation suggests that a gradual change occurs in the enzyme itself. This could be a spontaneous change in the protein alone, or a change resulting from gradual accumulation of metal ions present at very low concentration in the buffer solution, or possibly a gradual release of a bound inhibitor. Results with total extracts of *Lemna minor* (unpublished) show similar activation by storage or Zn^{2+}, indicating that this is not solely a property of root enzyme.

Regardless of the mechanism involved, it is likely that Zn^{2+} (or some related metal ion) plays an important role in the regulation of GDH activity. The inhibitory effect of some natural compounds such as citrate, and chelating agents added experimentally, can be explained by their direct effect on Zn^{2+} associated with the enzyme. Their inhibitory effect can be reversed by titration with Zn^{2+} or Ca^{2+}. For most of the agents tested (Table 4),

[15] BONE, D. H. (1959) *Nature* **184**, 990.
[16] RITENOUR, G. L., JOY, K. W., BUNNING, J. and HAGEMAN, R. H. (1967) *Plant Physiol.* **42**, 233.

the order of effectiveness as GDH inhibitor (EDTA > 8-hydroxyquinoline-sulphonate > citrate > oxalate) is in the same order as their effectiveness in binding Zn^{2+} (log stability constants for zinc complex: 16·4, 8·7, 6·2, 4·8 respectively; a value above 5 is indicative of a strong chelator[17]). Malate and succinate are ineffective in zinc chelation and are found to have no inhibitory effect. In a limited number of experiments, results with 1,10-phenanthroline were inconclusive. Both citrate and EDTA have been shown to be effective as inhibitors of GDH from micro-organisms[4–6] but in this case it is oxidative deamination which is inhibited, and under most conditions there is no effect on reductive amination. This is in direct contrast to the pea root enzyme which is inhibited principally in the direction of amination.[10] Other workers[8,11] have reported inhibition by EDTA and other chelating agents, of reductive amination by GDH from plant sources.

The partially inactivated enzyme freshly extracted from mitochondria shows substrate inhibition with high concentration of 2-oxoglutarate (Fig. 1a), and also to some extent with ammonia (Fig. 2) confirming the report by Chou and Splittstoesser.[9] The binding of 2-oxoglutarate at low concentrations appears to be similar in both partially inactive and activated enzymes. Citrate inhibition of active enzyme preparations is in this respect similar to the inactivation in fresh mitochondrial preparations (Fig. 1b). It is possible that citrate could be the agent causing the inactivation on extraction. However, it is not clear whether the enzyme is active *in vivo*, becoming inactivated by exposure to metabolites during extraction, or whether it is inactive in the mitochondria due to some regulatory action. The position of citrate in metabolism certainly does not make it a suitable substance for regulation of reductive amination. On the contrary, a high level of citrate should indicate an energy and carbon status quite favourable for glutamate synthesis, when GDH activity could be high. Therefore it may be that citrate does not play a natural role in GDH-regulation, and any effect it has is due to chelation or a related effect.

The data do not clearly indicate a mechanism to explain the various observations, particularly the effects of metal ions. Zinc appears to play a role in the animal (liver) enzyme which contains small amounts of the metal,[18] possibly being involved in binding together subunits into an active polymer. Excess zinc causes inactivation of the liver enzyme due to dissociation,[19] and zinc chelators also cause dissociation.[20] It could be that zinc (or another metal) is similarly involved in controlling the state of subunit aggregation of the plant enzyme. It was shown earlier that the activity of the pea root enzyme did not exhibit a linear response to increasing pyridine nucleotide concentration;[10] with the liver enzyme similar results are correlated with changes in the state of aggregation.[21] In the present work, the enzyme activity of a preparation of pea root GDH covalently bound to AE-cellulose, in which changes in degree of aggregation were presumably prevented, was unaffected by concentrations of citrate or EDTA which would significantly inhibit the free enzyme. This observation is consistent with the suggestion that the activation of free enzyme by Zn^{2+} and inactivation by chelating agents depends on an association–dissociation phenomenon. The speed of such activity changes are also consistent with this hypothesis, since it can take 30 sec or more for a new rate to be attained after addition of zinc or citrate. However, other

[17] In *Data for Biochemical Research*, (DAWSON, R. M. C., ELLIOTT, D. C., ELLIOT, W. H. and JONES, K. M., eds.), p. 423, Oxford University Press, Oxford (1969).
[18] VALLEE, B. L., ADELSTEIN, S. J. and OLSON, J. A. (1955) *J. Am. Chem. Soc.* **77**, 5196.
[19] TOMKINS, G. M., YIELDING, K. L., TALAL, N. and CURRAN, J. F. (1963) *Cold Spring Harb. Symp. Quant. Biol.* **28**, 461.
[20] FRIEDEN, C. (1957) *Biochim. Biophys. Acta* **27**, 431.
[21] FRIEDEN, C. (1959) *J. Biol. Chem.* **234**, 809.

explanations are possible. The metal ion may become attached to the enzyme protein without any change in state of aggregation, and chelators may simply remove the metal, or may cause inhibition by binding to the metallo-enzyme. Further work will be needed to elucidate this point.

Changes in enzyme activity caused by glutamate appear to be due to a mechanism different from that responsible for changes caused by metal ions. Glutamate alters the properties of the enzyme, including an increase in the apparent K_m for 2-oxoglutarate, and reversal of the substrate (2-oxoglutarate) inhibition of the freshly extracted enzyme. With high glutamate concentration in either the presence or absence of Zn^{2+} the final activity is about the same (Fig. 3). Sensitivity to glutamate is lost on purification but is recovered when the enzyme is immobilized. These changes are consistent with a conformational change in the enzyme mediated by glutamate acting as an allosteric effector. The activity of the immobilized enzyme shows, in the presence of glutamate, a pronounced sigmoid curve with increasing concentrations of 2-oxoglutarate, characteristics of an allosteric effect; however, it should be noted that the kinetics of immobilized enzymes may be distorted by diffusion and charge factors.[22] Further evidence for a glutamate mediated change of enzyme also comes from the immobilized GDH, showing that 2-oxoglutarate may partially offset the effect of glutamate. If glutamate is added to an assay system which already has an intermediate concentration of 2-oxoglutarate, there is only slight inhibition, much less than if glutamate had been present from the beginning of the assay run (Fig. 4). It appears as if the enzyme had been partially 'stabilized' in a more active form. This rules out any suggestion that glutamate is acting by simple competitive inhibition, or by altering the position of equilibrium of the reaction.

In most plant tissue, other than in germinating seeds and senescent material, GDH has a role predominantly involved in the synthesis of glutamate. The results suggest that glutamate can significantly influence its own rate of synthesis by GDH. NAD^+ also appears to regulate the activity of GDH (Fig. 4). Both glutamate and NAD^+ would be ideal substances to control NADH-dependent reductive amination, adapting the activity to glutamate requirements and to the energy status of the cell. Loss of sensitivity to glutamate during purification may be due to a loss of regulatory subunits, or more possibly to a gradual relaxation, so that a complete conformational change is no longer mediated by binding at an allosteric site. Attachment of the protein to an insoluble support may restore sufficient rigidity to allow glutamate to effect a change. D-Glutamate was in some respects an even stronger inhibitor of enzyme activity than L-glutamate, for both free and immobilized enzymes, although the effects were not completely comparable. Other amino acids tested have little regulatory effect on GDH. Since glutamate is involved in many different metabolic pathways, including transamination and carbon skeleton alteration to form other amino acids, glutamate itself rather than a subsequent end-product appears to provide the best regulatory information for its own synthesis.

It is interesting that the alteration of activity by both glutamate and zinc affect only NADH-dependent amination, NADPH activity being insensitive to both effectors. NAD^+-dependent deamination is insensitive to zinc (glutamate is of course a substrate for this reaction). This would suggest that these latter activities are not catalyzed by precisely the same reaction site or mechanism as is NADH activity. The fact that NADPH and NAD^+ activity may remain unchanged, while NADH activity alters, accounts for the changing ratio of NADH/NAD^+ activity noted previously.[10]

[22] SUNDARAM, P. V. and JOY, K. W. (1973) in preparation.

EXPERIMENTAL

Plant material. Pea roots (*Pisum sativum*, var. Little Marvel) were obtained from plants grown *ca.* 3 weeks in aerated nutrient solutions, with nitrate (8 mM) as the nitrogen source.

Enzyme extraction. Details of purification were essentially as described earlier,[10] involving precipitation at pH 3, ammonium sulphate fractionation (40–70%), and chromatography on Sephadex G200. For much of this work crude extracts were used, obtained by the following procedures carried out at 0–4°: (a) 'Total Homogenate' was obtained by grinding washed roots in 0·05 M Tris buffer, pH 7·5, containing 0·4 M sucrose, using 2·5 ml/g of tissue. After cheesecloth filtration, cell debris was removed by centrifugation for 5 min at 1000 *g*. When this fraction was to be used for assay, Tergitol NPX (Union Carbide) detergent was added to 0·1%, and the solution was clarified at 12 000 *g*. (b) Centrifugation of total homogenate (12 000 *g*, 10 min) yielded 'supernatant fraction' and a particulate (mitochondrial) sediment which was washed with grinding buffer and re-sedimented. This mitochondrial preparation was suspended in 0·1 M Tris, pH 8, containing 0·1% Tergitol NPX detergent, using a vol. *ca.* 0·25 of the fr. wt of roots originally homogenized; after 15 min an equal vol. of 0·2 M Tris, pH 8, was added and this crude 'mitochondrial fraction' was clarified by centrifugation.

Enzyme assay. Unless otherwise specified, the 'NADH assay' measuring the reductive amination of 2-oxoglutarate contained in 3 ml: Tris (pH 8·0), 133 mM; 2-oxoglutarate (neutralized), 13 mM; ammonium sulphate, 67 mM; NADH, 0·33 mM. The reaction was started by addition of NADH, and followed by recording oxidation of NADH at 340 nm at 25°. Where indicated zinc ions were added to the assay as the sulphate, at a final concentration of 0·3 mM. Controls were run lacking ammonia or 2-oxoglutarate. One unit of GDH catalyzes the oxidation of 1 μmol of coenzyme per min. Details of other assays have been reported earlier.[10]

Preparation of immobilized enzyme. Purified GDH was covalently bound to AE cellulose (N.B. Co), using glutaraldehyde as cross-linking agent at pH 9·0.[22] Assay solutions were continuously re-circulated through a column of the immobilized enzyme preparation, using a closed system containing a flow-through cell to follow oxidation of NADH spectrophotometrically. The system contained 20 ml of solution containing Tris, pH 7·8, 180 mM; ammonium sulphate, 200 mM; NADH, 0·5 mM; various concentrations of 2-oxoglutarate were added during the assay run. The immobilized preparation had an activity *ca.* 15% of that of the original purified free enzyme.

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