Insect Biochem., *Vol.* 12, *No.* 5, *pp.* 507–514, 1982.
Printed in Great Britain.

0020-1790/82/050507-08$03.00/0

PURIFICATION AND PROPERTIES OF GLUTAMATE DEHYDROGENASE FROM THE COLD-HARDY GALL FLY LARVA, *EUROSTA SOLIDAGINIS*

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(*Received* 11 *February* 1982)

Abstract—Glutamate dehydrogenase (GDH) from the goldenrod gall fly larva, *Eurosta solidaginis*, was purified 500-fold to a final specific activity of 37.5 μmol NADH utilized/min mg^{-1} protein. The enzyme is a hexamer of mol. wt 350,000 ± 30,000 and subunit size 57,000 ± 5000. Both nicotinamide coenzymes were utilized with activity ratios, NADH/NADPH, of 3.5 at pH 6.75 and 11 at pH 7.5; NAD/NADP activity ratios were 7 at pH 7.5 and 15 at pH 8. Enzyme kinetic constants ($S_{0.5}$) for NH_4^+, α-ketoglutarate and coenzyme were 210 ± 10, 2.7 ± 0.2 and 0.032 ± 0.001 mM for the NADH linked reaction and 61 ± 2.0, 0.56 ± 0.06 and 0.060 ± 0.003 mM for the NADPH reaction. Ammonium ion kinetics were distinctly sigmoidal. Metabolite effectors modified NH_4^+ kinetics, ADP activated, decreasing $S_{0.5}$ for NH_4^+ four-fold and reducing *n*, the Hill coefficient. ATP inhibited the NADH linked reaction but activated the NADPH reaction. GTP inhibited the enzyme. GDH activity in the forward, glutamate oxidizing, direction was undetectable in the absence of ADP. $S_{0.5}$ values for glutamate and coenzyme (at saturating ADP) were 3.1 ± 0.2 and 0.22 ± 0.02 mM for the NAD reaction and 3.8 ± 0.3 and 0.20 ± 0.02 mM for the NADP reaction. Inhibitors (GTP, ATP) modified both glutamate and coenzyme kinetics. Arrhenius plots were linear over the range 25–0°C and temperature had little effect on enzyme substrate affinity. Inhibitor effects, however, were altered with temperature; I_{50} values for GTP and ATP decreased at low temperature and ADP reversal of GTP inhibition of the NADPH linked reaction was greater at 5°C than at 25°C. Larval GDH appears well suited for a role in amino acid biosynthesis and may have an important role in the overwintering accumulation of proline in this freezing-tolerant insect.

Key Word Index: Eurosta solidaginis, glutamate dehydrogenase, cold hardiness, larval enzyme kinetics, low temperature enzyme function, enzyme regulation

INTRODUCTION

GLUTAMATE DEHYDROGENASE (GDH) (E.C. 1.4.1.3), catalyzing the reaction, L-glutamate + NAD(P) + $H_2O \rightleftharpoons$ α-ketoglutarate + NH_4^+ + NAD(P)H + H^+, is a key enzyme locus interrelating carbohydrate and amino acid metabolism in animals. The enzyme from mammalian sources utilizes either nicotinamide nucleotide with comparable facility while GDH from some invertebrate sources appears to be largely NAD specific (BURSELL, 1975; STOREY *et al.*, 1978). The role of GDH *in vivo* is still the subject of controversy. Early studies of GDH function in mammals suggested that the reaction was the major pathway of NH_4^+ production in nitrogen excretion (BRAUNSTEIN, 1957) while more recent work suggests a primary function for GDH in nitrogen storage through glutamate synthesis (PALAIOLOGOS and FELIG, 1976). It is possible that GDH has multiple roles in tissues, with its metabolic function closely regulated by substrate availability, choice of coenzyme utilized and the action of allosteric effectors such as ADP, ATP and GTP (GOLDIN and FRIEDEN, 1971). In insects, GDH may have both catabolic and anabolic functions and, in particular, may play a key role in the regulation of proline metabolism. GDH, in the forward direction, functions in the oxidation of proline, a major flight muscle fuel in many species, to introduce α-ketoglutarate, derived from proline, into the Krebs cycle (BURSELL, 1975). In the reverse direction, GDH is a pivotal locus in the *de novo* synthesis of many amino acids, including proline.

Amongst cold hardy insects, accumulations of proline and alanine often accompany low temperature acclimation (MANSINGH, 1967) and may have a cryoprotectant function. The larva of the goldenrod gall fly *Eurosta solidaginis* (Fitch), is freezing-tolerant and capable of surviving temperatures as low as −40°C during overwintering. The larvae accumulate high levels of glycerol (235 mM) and sorbitol (145 mM) as cryoprotectants as well as substantial amounts of proline (55 mM) during low temperature acclimation (STOREY *et al.*, 1981a). At low temperatures, proline synthesis is activated, a 50% increase in the total free amino acid pool during acclimation from 15 to −5°C resulting almost entirely from accumulation of proline. A transient increase in glutamate levels during this period suggests a precursor role for glutamate and the possible function of GDH as the site of incorporation of ammonium ion into the *de novo* production of this amino acid.

In the present investigation we have studied the kinetic and regulatory properties of purified GDH from *E. solidaginis* larvae. The study represents the first purification of the insect enzyme. Like many animal GDH's, but unlike adult insect (*Glossina mortisans*) flight muscle GDH (BURSELL, 1975), the larval form can make significant use of either coenzyme, NAD(H)

or NADP(H). Substrate affinities and modulator effects, by ADP, ATP and GTP, suggest that glutamate synthesis *in vivo* may be NADPH. linked and activity ratios, forward/reverse, for the larval enzyme favour the reverse (glutamate synthesizing) reaction much more strongly than does the enzyme from adult flight muscle. Temperature effects on ADP/GTP regulatory interactions may be important in activating the synthesis of glutamate, and accumulation of proline, during low temperature acclimation of the larvae.

MATERIALS AND METHODS

Animals and chemicals

Round galls containing third instar larvae of the gall fly, *Eurosta solidaginis*, were collected and acclimated to low temperature as previously described (STOREY *et al.*, 1981a). Larvae were then removed from their galls, killed by immersion in liquid nitrogen and stored at −80°C until use.

All biochemicals were purchased from Boehringer Mannheim Corp., phosphocellulose and mol. wt marker proteins were from Sigma Chemical Co. and Sephacryl S-300 was from Pharmacia Fine chemicals.

Purification of GDH

Whole larvae (3 g per preparation) were homogenized in 5 vol ice-cold 20 mM Tris–HCl buffer, pH 7.0 containing 114 mg/ml (20% saturation) ammonium sulphate using a Polytron PT 10-35 homogenizer at full speed for 3 × 5 sec. The homogenate was then centrifuged at 27,000 *g* for 30 min at 4°C. The supernatant was saved and the pellet was resuspended in a second volume of 20 mM Tris–HCl buffer, pH 7.0, rehomogenized and recentrifuged as above. The second supernatant was added to the first producing a crude enzyme preparation at 10% saturation with ammonium sulphate. Additional ammonium sulphate (183 mg/ml) was then added to bring the solution to 40% saturation. The solution was stirred at 23°C for 30 min, then centrifuged as above. The protein pellet was discarded and the supernatant was brought to 50% saturation (by addition of 63 mg/ml) with ammonium sulphate, stirred and centrifuged. The pellet, containing GDH, was resuspended in 15 ml 10 mM imidazole–HCl buffer, pH 6.5 containing 15 mM 2-mercaptoethanol and layered onto a phosphocellulose column (2 cm i.d. × 4 cm) equilibrated in the same buffer. The column was then washed with buffer until the A_{280} dropped to a minimum value. GDH was eluted from the column using a 0–4 M gradient of KCl run at 30 ml/hr. Peak fractions were pooled and concentrated to approximately 1 ml (to increase enzyme stability) using an Amicon Centriflo membrane cone with a mol. wt cut-off of 25,000. The concentrated enzyme was stable at 4°C for at least one week.

Mol. wt determination

Mol. wt determinations were performed using a column (50 × 0.7 cm) of Sephacryl S-300 with 20 mM imidazole–HCl buffer, pH 7.0 as the equilibration/elution buffer. Fractions (1 ml) were collected and assayed for GDH activity. Mol wt was determined from a plot of K_{av} vs log M_r for standard proteins: ferritin (M_r = 440,000), catalase (M_r = 240,000) and aldolase (M_r = 158,000).

SDS polyacrylamide gel electrophoresis

Electrophoresis using 10% slab gels was carried out at 40 mA constant current for 4 hr at 4°C using Tris–glycine (46 mM/35 mM); pH 8.0 containing 1% SDS as the electrode buffer. Prior to running, protein samples were preincubated with 1% SDS at 80°C for 20 min. Protein was detected using the silver stain of SWITZER *et al.* (1979). Subunit mol. wt was determined from a plot of R_f vs mol. wt for the protein standards: trypsinogen (24,000), ovalbumin (45,000) and bovine glutamate dehydrogenase (54,000).

Protein assay

Protein concentration was measured by the method of BRADFORD (1976) using the Bio-Rad Laboratories prepared reagent and bovine gamma globulin as the standard.

Enzyme assay and kinetics

GDH activity was monitored by following NAD(P) (H) utilization at 340 nm using a Pye Unicam SP 8-100 recording spectrophotometer with water jacketed cell holder for temperature control. Optimal assay conditions (final vol. = 1 ml) for GDH in the forward direction were: 50 mM Tris–HCl buffer, pH 8.0, 25 mM L-glutamate, 2 mM NAD(P) with 1 mM ADP for the NAD-linked activity and 0.5 mM ADP for the NADP linked activity. In the reverse direction, optimal conditions were: 50 mM imidazole–HCl buffer, pH 7.5, 300 mM NH_4Cl, 25 mM α-ketoglutarate 0.15 mM NADH and 0.5 mM ADP for the NADH-linked activity and 50 mM imidazole–HCl buffer, pH 6.75, 100 mM NH_4Cl, 5 mM α-ketoglutartate, 0.20 mM NADPH and 0.1 mM ADP for the NADPH linked activity. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol NADH per min at 25°C.

Kinetic constants, $S_{0.5}$ and n, were determined from Hill plots using experimentally measured V_{max} values determined at saturating substrate and cofactor concentrations. I_{50} values, the inhibitor concentration producing a 50% decrease in enzyme rate at constant substrate concentrations, were determined from plots of $V_{(uninhibited)}/V_{(inhibited)}$ against inhibitor concentration by the method of JOB *et al.* (1978).

For low temperature studies, pH of buffers was adjusted at 25°C and buffer pH was allowed to change with changing temperature (for imidazole this results in an approximately 0.017 pH unit increase in pH per 1°C decrease in temperature (SOMERO, 1981) mimicking *in vivo* pH change with temperature. It is now well known that intracellular pH of ectotherms varies with environmental temperature in a manner which preserves the fractional dissociation state of histidine imidazolium groups at a constant value (SOMERO, 1981).

RESULTS

Purification and physical properties

The maximal activity of GDH in *E. solidaginis* was 6.7 units/g wet wt of larvae. Previously we reported about three units of GDH in the larvae (STOREY and STOREY, 1981); yield in the present study was greatly improved by the inclusion of ammonium sulphate in the homogenizing medium. The purification of *E. solidaginis* GDH is summarized in Table 1. Cellulose phosphate provided a very high purification (290 fold) of the enzyme in a single step. The final preparation was purified about 500 fold to a final specific activity of 37.5 units/mg protein compared to specific activities of 40 units/mg for ox liver and brain GDH (MCCARTHY *et al.*, 1980) and 43–50 units/mg for commercial bovine liver GDH from Sigma Chemical Co., The enzyme was judged to be >95% pure by SDS electrophoresis.

Mol. wt of *E. solidaginis* GDH was found to be 350,000 ± 30,000 (n = 7) similar to an average mol. wt of about 330,000 reported for most species (SMITH *et al.*, 1975). Subunit size was 57,000 ± 5000 (n = 4) suggesting that the enzyme, like all other animal GDH's, is a hexamer.

Table 1. Purification of glutamate dehydrogenase from the gall fly larva, *Eurosta solidaginis*

Step	Total activity (units)	Total protein (mg)	Yield (%)	Purification (fold)	Specific activity (units/mg protein)
Crude homogenate	17.9	244	—	—	0.073
Ammonium sulphate	15.0	115	84	1.8	0.130
Cellulose phosphate	9.0	0.24	50	512	37.5

pH optima and coenzyme utilization

Figure 1 shows pH profiles for *E. solidaginis* GDH. In the reverse direction (NAD(P)H utilizing), the enzyme has pH optima of 7.5 and 6.75 for the NADH and NADPH reactions, respectively while in the forward direction, the optima are 8.0 and 7.5 for NAD and NADP, respectively. The acidic shift in the pH optimum in the presence of NADP or NADPH was also reported for the *Drosophila* larval enzyme (BOND and SANG, 1968). Enzyme activity with NAD(H) as the coenzyme was significantly higher than that with NADP(H). In the reverse direction, the activity ratio, NADH/NADPH was 11 at pH 7.5 but dropped to 3.5 at pH 6.75. In the forward direction, the ratio NAD/NADP was 15 at pH 8 and 7 at pH 7.5.

The ratio of enzyme activity in the forward vs reverse directions (at the respective pH optima) was 1:10 for NAD/NADH and 1:20 for NADP/NADPH.

The reverse reaction

Substrate affinities. Tables 2 and 3 show the substrate affinities ($S_{0.5}$) and Hill coefficients (n) for *E. solidaginis* GDH for the NADH and NADPH linked reactions, respectively. Enzyme affinity for both NH_4^+ and α-ketoglutarate was significantly higher ($S_{0.5}$ values four- and five-fold lower, respectively) for the NADPH linked reaction vs the NADH linked reaction. The lower $S_{0.5}$ values for the NADPH reaction probably more closely resemble *in vivo* levels of these substrates, perhaps suggesting that NADPH is the preferred coenzyme for the reductive amination reaction of GDH. The $S_{0.5}$ for NADH is, however, about two-fold lower than the affinity constant for NADPH. Ammonium ion kinetics are strongly sigmoidal in the presence of either coenzyme with values for n of about three.

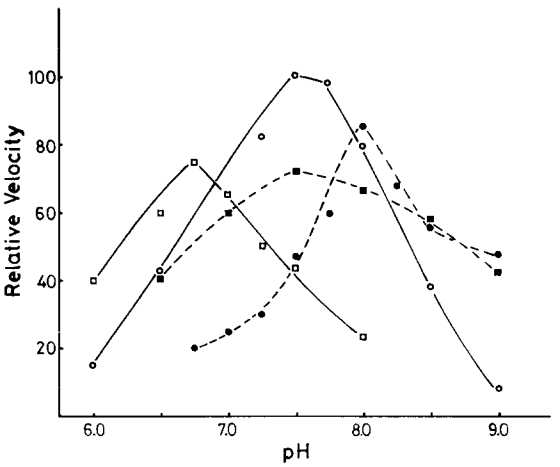

Fig. 1. pH profiles for *E. solidaginis* GDH. Enzyme activity was measured at 25°C under optimal assay conditions as outlined in Materials and Methods. The pH profile for each coenzyme is expressed as a relative activity, the average of $n = 3$ determinations with reproducibility ±10%. Symbols are: for the forward reaction: ●, NAD; ■, NADP; for the reverse direction: ○, NADH; □, NADPH.

ADP effects. ADP activated both the NADH and the NADPH linked reactions. For the NADH reaction, maximal activation was 143% of control activity and occurred at 0.5 mM ADP. Maximal activation of the NADPH linked reaction was 138% at 0.05 mM ADP. ADP effects on enzyme velocity for the *E. solidaginis* enzyme are considerably less than those noted for the enzyme from other sources, activation by ADP being about four-fold for bovine liver GDH (GOLDIN and FRIEDEN, 1971) and as high as 100-fold for squid muscle GDH (STOREY *et al.*, 1978). Half maximal activation by ADP occurred at 0.1 and 0.01 mM ADP for the NADH and NADPH linked reactions, respectively. For the NADPH reaction, ADP activation occurred at levels well within the psysiological concentration range of ADP in *E. solidaginis* (STOREY *et al.*, 1981a).

Modifier effects on substrate affinities. Table 2 shows the effects of metabolite modulators on substrate affinities of the NADH linked reaction. The activator, ADP, and the inhibitors, ATP and GTP, had no significant effects on the $S_{0.5}$ or n for either α-ketoglutarate or NADH. All three modulators strongly affected ammonium ion kinetics. The addition of 0.5 mM ADP decreased $S_{0.5}$ for NH_4^+ by four-fold and reduced n by one-half (producing a more hyperbolic substrate saturation curve). The inhibitors, ATP and GTP, however, increased the $S_{0.5}$ for NH_4^+ by two-fold (over control values) and also doubled the n value. ADP added along with either ATP or GTP produced a partial reversal of ATP and GTP inhibitions lowering both $S_{0.5}$ for NH_4^+ and n.

The $S_{0.5}$ for NH_4^+ was also affected by assay pH, decreasing as pH increased from 6.0 to 8.0 but then increasing as pH rose farther. $S_{0.5}$ for NH_4^+ was 170, 130, 90, 50, 40 and 60 mM at pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively for the NADH linked enzyme assayed in the presence of 0.5 mM ADP. A similar effect of pH on ammonium ion affinity was reported for the *Drosophila* larva enzyme by BOND and SANG (1968) but has not been reported for other GDH's.

Metabolite modulator effects on the NADPH linked reaction of GDH were primarily confined to effects on NH_4^+ kinetics (Table 3) although GTP inhibition of the enzyme also increased $S_{0.5}$ for both

Table 2. Kinetic constants for the NADH-linked reaction of *E. solidaginis* glutamate dehydrogenase: Effects of activators and inhibitors

Addition	NH_4Cl		α-Ketoglutarate		NADH	
	$S_{0.5}$	n	$S_{0.5}$	n	$S_{0.5}$	n
Control	210 ± 10	3.5 ± 0.05	2.7 ± 0.2	1.2 ± 0.1	0.032 ± 0.001	1.5 ± 0.1
ADP, 0.5 mM	52 ± 2	1.6 ± 0.1	2.3 ± 0.1	1.3 ± 0.1	0.027 ± 0.001	1.5 ± 0.1
ATP, 2 mM	400 ± 13	7.3 ± 0.5	2.3 ± 0.2	1.1 ± 0.1	0.030 ± 0.003	1.7 ± 0.1
GTP, 20 μM	440 ± 26	7.6 ± 0.8	2.8 ± 0.03	1.3 ± 0.1	0.028 ± 0.003	1.7 ± 0.2
ADP, 0.5 mM plus ATP, 2 mM	300 ± 16	5.1 ± 0.6				
ADP, 0.5 mM plus GTP, 20 μM	370 ± 18	6.5 ± 0.6				

$S_{0.5}$ values are in mM ± S.E.M., from determinations on at least three different enzyme preparations.

Kinetic constants were derived from Hill plots using V_{max} measured at substrate concentrations: 25 mM α-ketoglutarate, 0.15 mM NADH and 450 mM (control), 300 mM (plus ADP) or 600 mM (all other additions) NH_4Cl. Assays were performed in 50 mM imidazole buffer, pH 7.5 at 25°C.

Table 3. Kinetic constants for the NADPH-linked reaction of *E. solidaginis* glutamate dehydrogenase: effects of activators and inhibitors

Addition	NH_4Cl		α-Ketoglutarate		NADPH	
	$S_{0.5}$	n	$S_{0.5}$	n	$S_{0.5}$	n
Control	61 ± 2.0	2.8 ± 0.1	0.56 ± 0.06	1.1 ± 0.1	0.060 ± 0.003	1.7 ± 0.2
ADP, 0.1 mM	15 ± 1.0	1.4 ± 0.1	0.51 ± 0.03	1.1 ± 0.1	0.062 ± 0.003	1.3 ± 0.1
ATP, 2 mM	12 ± 1.0	1.1 ± 0.2	0.54 ± 0.06	1.2 ± 0.02	0.063 ± 0.001	1.3 ± 0.2
GTP, 2 μM	110 ± 8.0	7.8 ± 0.9	1.3 ± 0.1	1.3 ± 0.1	0.074 ± 0.003	1.8 ± 0.1
ADP, 0.1 mM plus	19 ± 0.03	1.1 ± 0.2				
ATP, 2 mM plus GTP, 2 μM	11 ± 2.0	1.1 ± 0.01				

$S_{0.5}$ values are in mM ± S.E.M. from determinations on at least three different enzyme preparations.

Kinetic constants were derived from Hill plots using V_{max} measured at the following substrate concentrations: 0.20 mM NADPH, 5 mM α-ketoglutarate (7.5 mM for GTP alone) and 100 mM (ADP or ATP), 180 mM (GTP) or 120 mM (control + all others) NH_4Cl. Assays were performed in 50 mM imidazole buffer pH 6.75 at 25°C.

α-ketoglutarate and NADPH. As for the NADH linked reaction, ADP decreased the $S_{0.5}$ for NH_4^+ of the NADPH reaction by four-fold and reduced n by one-half. ATP effects on the NADPH linked reaction are opposite to those of the metabolite on the NADH reaction. ATP activates the NADPH reaction, decreasing $S_{0.5}$ by five-fold and strongly reducing n to produce hyperbolic kinetics. The major effect of GTP inhibition on the NADPH linked reaction was a two-fold increase in $S_{0.5}$ for NH_4^+ with a corresponding increase in n similar to the effects of GTP on the NADH linked enzyme. Unlike the NADH linked reaction, however, GTP inhibition of the enzyme was completely overridden by the addition of ADP and also by addition of ATP, $S_{0.5}$ for NH_4^+ being reduced to the level of that in the presence of ADP alone.

The forward reaction

Substrate affinities and ADP effects. Activity of *E. solidaginis* GDH in the forward (glutamate oxidizing) direction is completely dependent upon the presence of ADP with no enzyme activity detected in the absence of this activator. Maximal enzyme activity occurred with the addition of 1.0 or 0.5 mM ADP for

Table 4. Kinetic constants for the NAD linked reaction of *E. solidaginis* glutamate dehydrogenase: Effect of modifiers

Addition	Glutamate		NAD	
	$S_{0.5}$	n	$S_{0.5}$	n
ADP, 1 mM	3.1 ± 0.2	1.0 ± 0.03	0.22 ± 0.02	1.1 ± 0.1
ADP, 1 mM plus 0.1 mM ATP	0.90 ± 0.01	1.2 ± 0.2	0.031 ± 0.002	1.0 ± 0.1
ADP, 1 mM plus GTP, 1.5 μM	1.5 ± 0.1	1.6 ± 0.2	0.018 ± 0.001	1.1 ± 0.2

$S_{0.5}$ values are in mM ± S.E.M. and are the average of determinations on at least three different enzyme preparations. Kinetic constants were derived from Hill plots with V_{max} measured at the following substrate concentrations: ADP alone, 25 mM glutamate, 2 mM NAD; ADP + ATP, 12.5 mM glutamate, 0.6 mM NAD; and ADP + GTP, 20 mM glutamate, 0.4 mM NAD. Assays were performed in 50 mM Tris–HCl buffer, pH 8.0 at 25°C.

Table 5. Kinetic constants for the NADP linked reactions of *E. solidaginis* glutamate dehydrogenase: effect of modifiers

Addition	Glutamate		NADP	
	$S_{0.5}$	n	$S_{0.5}$	n
ADP, 0.5 mM	3.8 ± 0.3	1.4 ± 0.1	0.20 ± 0.02	1.2 ± 0.04
ADP, 0.5 mM plus ATP, 0.5 mM	3.3 ± 0.2	1.5 ± 0.2	0.058 ± 0.003	1.0 ± 0.1
ADP, 0.5 mM plus GTP, 4 μM	2.9 ± 0.4	1.4 ± 0.3	0.061 ± 0.013	1.1 ± 0.02

$S_{0.5}$ values are in mM ± S.E.M. and are the average of determinations on at least three different enzyme preparations. Kinetic constants were derived from Hill plots with V_{max} measured at the following substrate concentrations: ADP alone, 25 mM glutamate, 2 mM NADP, all others, 20 mM glutamate, 1 mM NADP. Assays were performed in 50 mM Tris–HCl buffer, pH 8.0 at 25°C.

the NAD and NADP linked reactions, respectively. The kinetic constants for glutamate, NAD and NADP are shown in Tables 4 and 5. Unlike the reverse reaction, the NADP and NADP linked reactions showed similar kinetic constants for both glutamate and coenzyme. The $S_{0.5}$ for glutamate was 3.1 (for the NAD reaction) and 3.8 mM (for NADP) similar to the K_m for glutamate of the *Drosophila* larval enzyme and well within *in vivo* levels of glutamate in *E. solidaginis* (3–6 μmol/g wet wt (Storey *et al.*, 1981a)).

Effect of modifiers. The NAD linked reaction was inhibited by ATP and GTP, both of which decreased enzyme velocity by approximately 50% over control (ADP activated) activity. However $S_{0.5}$ for both glutamate and NAD were also reduced in the presence of ATP or GTP (Table 4) showing that the inhibitors act in an uncompetitive manner. ATP produced a three-fold decrease in $S_{0.5}$ for glutamate and a seven-fold decrease in $S_{0.5}$ for NAD. GTP inhibition had somewhat greater effects on $S_{0.5}$ for NAD, decreasing coenzyme affinity by 12 fold.

ATP and GTP had little effect on glutamate affinity of the NADP linked reaction but both decreased the $S_{0.5}$ for NADP by about three-fold. Inhibition, again, was of the uncompetitive type.

Inhibitor constants

Table 6 shows the inhibitor constants for ATP and GTP for the forward and reverse reactions of *E. solidaginis* GDH. In the reverse direction, the inhibitor constant for GTP was much lower for the NADPH linked activity than for the NADH linked activity suggesting stronger control by the guanylates on NADPH linked GDH activity.Reversal of GTP inhibition by ADP increased the I_{50} for GTP two-fold for the NADH linked reaction and 7.5 fold for the NADPH linked reaction. GTP inhibition of the forward reaction was significantly more potent than that of the reverse reaction with I_{50} values about ten-fold lower than the corresponding I_{50} values for the reverse reaction.

ATP effects on the NAD and NADH reactions followed a similar pattern. For the NADH linked activity, the I_{50} for ATP (2.5 mM) is similar to intracellular ATP levels in *E. solidaginis* (Storey *et al.*, 1981a) but in the presence of ADP, I_{50} increases significantly. The I_{50} for ATP in the forward direction is about 50 fold lower than the corresponding I_{50} for the reverse reaction indicating a much tighter control of the forward, glutamate-oxidizing, reaction by high cellular energy levels, a factor which may be very important in regulating the rates of the forward vs reverse reactions *in vivo*.

Temperature effects on GDH

Figure 2 (a & b) shows Arrhenius plots for *E. solidaginis* GDH. The plots are linear over the range 25–

Table 6. Inhibitor constants (I_{50}) for ATP and GTP for *E. solidaginis* glutamate dehydrogenase

Addition	Coenzyme linked reaction					
	NADH		NADPH		NAD	NADP
	25°	5°	25°	5°	25°	25°
	I_{50}(mM)					
ATP	2.5 ± 0.3	0.88 ± 0.15	–	–	n.a.	n.a.
ATP + saturating ADP	6.2 ± 0.4	2.5 ± 0.1	–	–	0.11 ± 0.01	
	I_{50} (μM)					
GTP	19 ± 1.0	4.4 ± 0.5	1.7 ± 0.2	0.81 ± 0.08	n.a.	n.a.
GTP + saturating ADP	38 ± 0.4	9.9 ± 0.9	13 ± 1.0	27 ± 2.0	1.4 ± 0.1	4.3 ± 0.8

I_{50} values were determined by the method of Job *et al.* (1978) at the following substrate concentrations: for NADH: 0.15 mM NADH, 450 mM NH_4Cl (300 mM in the presence of 0.5 mM ADP), and 25 mM (25°C) or 40 mM (5°C) α-ketoglutarate; for NADPH 0.2 mM NADPH, 120 mM NH_4Cl (100 mM with 0.1 mM ADP), and 5 mM (25°C) or 15 mM (5°C) α-ketoglutarate; for NAD: 2.0 mM NAD, 25 mM L-glutamate and 1 mM ADP; and for NADP: 2.0 mM NADP, 25 mM L-glutamate and 0.5 mM ADP.

Values are means ± S.E.M. for at least three determinations on separate preparations. n.a.—no activity in the absence of ADP.

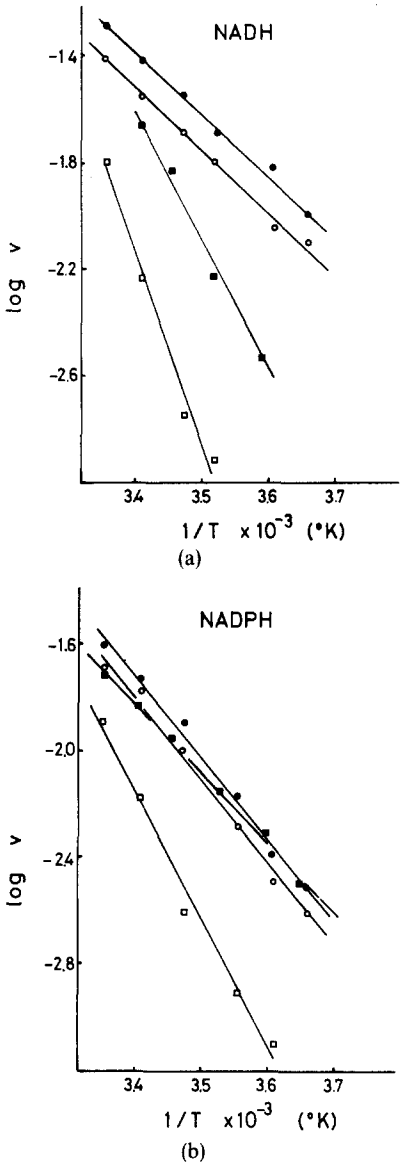

Fig. 2. Arrhenius plots for *E. solidaginis* GDH. Enzyme activity was measured under optimal assay conditions as outlined in Materials and Methods with the addition of ADP (0.5 or 0.1 mM for NADH vs NADPH reactions) or GTP (20 or 2 μM for NADH vs NADPH). A. NADH linked reaction. B. NADPH linked reaction. Symbols are: ○, control (minus ADP); ●, plus ADP; □, plus GTP; ■, plus ADP and GTP.

0°C. For both the NADH and NADPH reactions, in the absence of modifiers, calculated activation energy was 12,540 cal/mole with a Q_{10} of about 2. ADP, while activating enzyme activity, did not affect the slope of the Arrhenius plot. GTP, however, sharply increased the slope of the line suggesting stronger inhibition of GDH by GTP as temperature decreases. In the presence of both GTP and ADP, the slope of the Arrhenius plot returned to control levels for the NADPH reaction but was only partially reversed for the NADH reaction. The addition of glycerol (0.5 M) or sorbitol (0.25 M) or both, the polyols accumulated by *E. solidaginis* during overwintering, had no effect on the slope of the Arrhenius plot.

Temperature effects on GDH kinetic constants were compared at 25 and 5°C to determine whether temperature affected enzyme properties. $S_{0.5}$ for NADH and NADPH as well as for NH_4^+ (with either coenzyme and ±ADP) were found to be the same at both temperatures. Enzyme affinity for α-ketoglutarate, however, decreased significantly at the lower temperature. For the NADH linked enzyme, $S_{0.5}$ for α-ketoglutarate increased from 2.7 mM at 25°C (Table 2) to 4.1 ± 0.3 mM at 5°C. $S_{0.5}$ for α-ketoglutarate for the NADPH linked enzyme also increased with decreasing temperature from 0.56 mM at 25°C (Table 3) to 1.5 ± 0.1 mM at 5°C. Enzyme affinity for α-ketoglutarate at either temperature was unaltered by the addition of ADP.

The major difference in GDH kinetics at 5 vs 25°C was in the effects of inhibitors, both ATP and GTP showing more potent inhibition of the enzyme at low temperature. Inhibitor constants for ATP and GTP at 5°C are shown in Table 6 along with those for 25°C. For ATP, I_{50} decreased about three-fold at 5°C compared to 25°C. I_{50} values for GTP were also reduced with a greater reduction in I_{50} (four-fold) seen for GTP effects on the NADH linked enzyme than for the NADPH linked enzyme (two-fold). This decrease in I_{50} with decreasing temperature produces the effect of GTP on the Arrhenius plot seen in Fig. 2; at constant GTP concentration, enzyme activity is inhibited to a greater percentage as temperature decreases. ADP raises the I_{50} for both GTP and ATP at both temperatures. The effect is an approximate 2–2.5 fold increase in I_{50} for the NADH linked enzyme at 5°C, similar to the effect at 25°C. GTP/ADP interactions for the NADPH linked enzyme were differentially effected by temperature, however. At 25°C, ADP reversal of GTP inhibition resulted in a 7.5-fold rise in I_{50} to 13 μM while at 5°C ADP effects raised the I_{50} for GTP to 27 μM, a 34-fold increase. This effect could be important in allowing GDH function (and proline synthesis) at low temperatures.

DISCUSSION

Glutamate dehydrogenase from *E. solidaginis* larvae, like most animal GDH's, (a) is a hexamer of mol. wt 350,000, (b) occurs in a single form with no evidence for isozymes and no alteration of enzyme form during low temperature acclimation (STOREY *et al.*, 1981b), (c) displays a dual nucleotide specificity utilizing both NAD(H) and NADP(H) and (d) is a regulatory enzyme showing allosteric effects by the modulators, ADP, ATP and GTP. Some kinetic properties of insect larval GDH were previously studied by BOND and SANG (1968) using an unpurified and highly unstable ($t_{0.5}$ = 10 hr) GDH preparation from *Drosophila* larvae. The present study, utilizing the purified and stable GDH preparation from *E. solidaginis*, provids a more complete kinetic analysis of the enzyme. The two enzymes share some common properties including a strong effect of pH on enzyme affinity for ammonium ion, similar activity ratios, NAD/NADP and NADH/NADPH, and similar enzyme affinities for substrates.

While strongly controlled by allosteric modulations, GDH from different sources can also be poised for differential function in oxidative deamina-

tion (NH_4^+ excretion, oxidation of amino acids for energy production) or reductive amination (amino acid synthesis, nitrogen storage) through adjustments in the relative rates of the NAD(H) vs NADP(H) reactions (SMITH *et al.*, 1975) and the relative rates of the forward (NAD(P) utilizing) vs reverse (NAD(P)H utilizing) reactions. The activity ratio forward:reverse for *E. solidaginis* GDH was 1:10 for the NAD(H) reaction compared to 1:15 for bovine liver, 1:6, for insect flight muscle and 1:1 for squid mantle muscle GDH (STOREY *et al.*, 1978). GDH function in the latter two tissues is involved in glutamate (derived from proline) oxidation as a fuel for muscle work and appears to be poised for this function through a relatively high activity of the forward reaction compared to the reverse. Compared to the adult flight muscle GDH, larval GDH has an activity ratio favouring the reverse reaction, similar to that of bovine liver GDH, and perhaps suggesting that the larval enzyme, vs the adult flight muscle enzyme, is more strongly poised for a role in glutamate synthesis than in glutamate oxidation. Our observations of the accumulation of proline by *de novo* synthesis of the amino acid, during low temperature acclimation of *E. solidaginis* (STOREY *et al.*, 1981a), shows that a major function of *E. solidaginis* GDH is probably the synthesis of amino acids.

The relative rates of the NAD vs NADP and NADH vs NADPH reactions also vary between GDH from different sources. The activity ratio NADH/NADPH is 3.5 (at pH 6.75) for *E. solidaginis* GDH identical to that for the *Drosophila* larva enzyme (BOND and SANG, 1968). GDH from adult insect flight muscle, however is highly NAD(H) specific (BURSELL, 1975; MILLS and COCHRAN, 1963), BURSELL (1975) reporting an NADPH activity less than 2% of the NADH activity. GDH from squid mantle muscle and from various lower vertebrate species also shows very low activity with NADPH but the bovine liver enzyme utilizes the two coenzymes at almost equal rates (STOREY *et al.*, 1978; SMITH *et al.*, 1975). In mammalian systems, the NADPH linked activity of GDH is believed to be associated with glutamate synthesis *in vivo*; studies with perfused liver or isolated liver cells suggest that the primary source of reducing power for the reductive amination of α-ketoglutarate, stimulated by ammonium ion, is NADPH (SIES *et al.*, 1974, 1975; TISCHLER *et al.*, 1977). Extending this analogy to insect larval vs adult flight muscle GDH, it could be suggested that the relatively high NADPH linked activity shown by larval GDH underlies a major role in amino acid synthesis for the larval enzyme, a function which appears to be NADPH linked.

The kinetic properties of the reverse reaction of *E. solidaginis* GDH also suggest that the NADPH linked reaction may be favoured *in vivo*. $S_{0.5}$ for α-ketoglutarate is five-fold lower for the NADPH linked enzyme than for the NADH linked reaction and at 0.56 mM is perhaps within the physiological concentration range of this compound *in vivo*. $S_{0.5}$ for NH_4^+ is similarly 3.5-fold lower for the NADPH linked enzyme and, particularly for the ADP-activated NADPH linked enzyme, is probably within physiological ammonium ion concentrations. GDH's with dual nucleotide specificities probably preferentially utilize the NADPH linked function for glutamate biosynthesis. Enzymes which are highly specific for NAD(H), however, perhaps permit NADH linked glutamate synthesis through modifications lowering the affinity constants for α-ketoglutarate ($S_{0.5}$ for α-ketoglutarate for the NADH linked enzyme is 2.7, 0.8 and 0.35 mM for *E. solidaginis*, insect flight muscle and squid mantle muscle GDH, respectively (BURSELL, 1975; STOREY *et al.*, 1978)).

In the forward direction, enzyme affinities for glutamate and coenzyme were similar for both the NAD and NADP linked reactions. However, the activity ratio, NAD/NADP was higher at all pH values than the ratio NADH/NADPH for the reverse reaction. This may suggest that the forward reaction is linked preferentially to NAD. Modifier effects on the forward reaction also suggest this result (see below). However, while enzyme kinetic properties may suggest a coenzyme preference by GDH, it must be remembered that coenzyme utilization by GDH *in vivo* is probably largely governed by the mitochondrial availability of NAD vs NADP or NADH vs NADPH while the direction of GDH function (forward vs reverse) is partially determined by mitochondrial redox ratios.

The effects of metabolite modulators on *E. solidaginis* GDH are similar to those reported for GDH from other sources: ADP activates the enzyme, GTP inhibits and ATP is an inhibitor of the NAD(H) linked reaction but an activator of the NADP(H) reaction. Like GDH from a variety of invertebrate sources (BURSELL, 1975; STOREY *et al.*, 1978), enzyme activity in the forward direction is virtually undetectable in the absence of ADP. In the reverse direction, ADP activates enzyme activity but the effects of ADP on enzyme velocity for *E. solidaginis* GDH (about 140% activation at optimal substrate levels) are much lower than the effects of ADP on the insect flight muscle enzyme (a 20-fold activation) (BURSELL, 1975) and more closely resemble the four-fold activation by ADP reported for bovine liver GDH. ADP had strong effects on enzyme affinity constants, however. In the reverse direction, ADP produced a four-fold decrease in $S_{0.5}$ for ammonium ion for both the NADH and NADPH. For the NADPH linked reaction, this effect occurred not only at ADP levels (0.1 mM) which are physiological (STOREY *et al.*, 1981a) but also lowered enzyme affinity for NH_4^+ into a range which may be physiological.

GTP inhibition of GDH is extremely potent. I_{50} values for the forward reaction occurred in the range 1–4 μM while those for the reverse reaction were about 10-fold higher. ATP inhibition of the NAD(H) linked reactions is similarly much stronger (I_{50} about 50 times lower) for the forward direction. Oxidation of glutamate (or other amino acids which are catabolized via glutamate) is, therefore, strongly controlled by cellular energy status, mobilization of amino acid stores as metabolic fuels occurring only in low energy states. Glutamate synthesis (for amino acid and protein production), however should occur during high energy, growth states consistent with the lower inhibitory effects of GTP and ATP on the reverse reaction and the activating effects of ATP on NADPH linked glutamate synthesis.

Temperature change had minimal effects on *E. solidaginis* GDH. No abnormal effect of low temperature

on enzyme activity was seen: Arrhenius plots were linear and Q_{10} was 2. Enzyme function in this cold hardy larva appears to be controlled and co-ordinated over a wide temperature range, a feature which is well suited for maintaining metabolic control during large daily and seasonal ambient temperature changes. The production of proline (a possible cryoprotectant) in the larvae during low temperature acclimation (between 15 and −5°C) (STOREY *et al.*, 1981a) may be favoured by low temperature alterations in ADP/GTP modifier interactions on *E. solidaginis* GDH. GTP inhibition of NADPH linked glutamate synthesis is much more strongly reversed by low (physiological) levels of ADP at low temperature (5°C) than at high temperature (25°C). This effect could be responsible for increased synthesis of glutamate leading to proline accumulation as acclimation temperature decreases.

Acknowledgement—Supported by an N.S.E.R.C. operating grant to KBS.

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