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Tissue Specific Isozymes of Glutamate Dehydrogenase from the Japanese Beetle, *Popillia japonica*: Catabolic vs Anabolic GDH's

Keith B. Male and Kenneth B. Storey
Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

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Summary. 1. Glutamate dehydrogenase (GDH) from the Japanese beetle, *Popillia japonica,* occurs in tissue specific isozymic forms. Two forms, specific for flight muscle and fat body, were identified and were separable by starch gel electrophoresis and by differential elution from NAD-agarose.

2. The isozymes utilized both NAD(H) and NADP(H) as coenzymes with activity ratios NADH:NADPH of 6:1 for flight muscle and 8:1 for fat body. pH optima for both enzymes were similar.

3. GDH from the two tissues differed kinetically. Affinity for α-ketoglutarate was much higher for the fat body enzyme, $S_{0.5}$ for the NADH and NADPH linked reactions being 0.81 ± 0.09 and 0.26 ± 0.03 mM for fat body and 2.4 ± 0.03 and 2.3 ± 0.4 mM for flight muscle GDH, respectively.

4. Flight muscle GDH was much more strongly regulated by nucleotides than was the fat body isozyme. The apparent activation constant, K_a, for ADP was 2–3 fold lower for the flight muscle enzyme for both forward and reverse reactions and ADP had a greater effect in lowering $S_{0.5}$ for NH_4^+ for flight muscle GDH. GTP was a strong inhibitor of flight muscle GDH with apparent inhibitor constants, I_{50}, of 15.5 ± 3.0, 4.0 ± 0.9 and 6.5 ± 0.9 μM for the NADH, NADPH and NAD linked reactions, respectively. Fat body GDH, however, was only weakly affected by GTP with an I_{50} of 60 ± 6 μM for the NAD reaction and I_{50}'s of greater than 500 μM for the NADH and NADPH linked reactions.

5. The kinetic properties of the two GDH isozymes suit the probable roles of the enzyme in vivo. Flight muscle GDH has a major role in the oxidation of proline as a fuel for flight. Nucleotide control of GDH would allow enzyme activity to respond to the energy status of the cell and would achieve a rapid activation of GDH at the initiation of flight. Fat body GDH, however, has a major role in the biosynthesis of proline and other amino acids. Enzyme activity is probably regulated by substrate availability, the absence of strong nucleotide regulation allowing enzyme function in high energy, resting states.

Introduction

Glutamate dehydrogenase (GDH) (E.C. 1.4.1.3), catalyzing the reaction

$$\text{L-glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NAD(P)H} + \text{H}^+,$$

is a key enzyme locus interrelating carbohydrate and amino acid metabolism in the cell. The enzyme functions in both catabolic and anabolic roles in vivo. In the forward direction GDH plays a major role in nitrogen excretion (Braunstein 1957) and in the oxidation of amino acids (such as proline) as metabolic energy sources (Bursell 1975; Hansford and Johnson 1975). In the reverse direction, the enzyme is the major site for the incorporation of ammonium ion in the synthesis of amino acids (McGiven and Chappell 1975). Regulation of GDH is vested in a number of controls including substrate availability, choice of coenzyme utilized, allosteric effects by ATP, GTP, ADP and leucine and reversible polymerizations of subunits (Goldin and Frieden 1971; Smith et al. 1975). To date, however, the evidence has suggested that only a single molecular form of GDH is present in animal tissues; tissue specific, kinetically differing forms of the enzyme have not been found. However, distinct molecular forms of GDH occur in the mitochondria versus chloroplasts of plants (McKenzie

et al. 1981) and amongst yeasts (Hemmings 1978, 1980).

The present study provides the first demonstration of the occurrence of isozymic forms of glutamate dehydrogenase in animal tissues. Tissue specific forms of GDH were found in the flight muscle and fat body of the Japanese beetle, *Popillia japonica*. These enzymes, which were separable by starch gel electrophoresis and affinity chromatography on NAD-agarose, showed distinct kinetic differences. The kinetic properties of the isozymes appear to poise the enzymes for tissue specific functions, the muscle enzyme for the oxidation of proline as a fuel for flight and the fat body enzyme for the biosynthesis of amino acids including proline.

Materials and Methods

Animals and Chemicals

Male Japanese beetles, *Popillia japonica,* were obtained from Dr. M. Klein, Japanese Beetle Research Institute, Wooster, Ohio. All biochemicals were purchased from Boehringer Mannheim Corp., NAD-agarose was from P & L Biochemicals, Inc.

Enzyme Preparation

Beetles were immobilized by chilling on ice and were then killed by decapitation. Flight muscle and fat body were dissected out with care taken to remove the gut. Tissues were homogenized in 20 mM imidazole buffer, pH 7.5 using a glass-glass homogenizer. Homogenates were centrifuged at 27,000 *g* for 20 min at 4 °C. The resulting supernatant, without further preparation, was used for electrophoresis and chromatography studies; for kinetic studies the crude supernatant was first passed through a column of Sephadex G-100 (2 × 5 cm) to remove low molecular weight metabolites.

Starch Gel Electrophoresis

Flight muscle and fat body samples were applied to starch gels and run using the Ridgeway system consisting of lithium borate (60 mM lithium hydroxide, 300 mM boric acid) pH 8.2 as the electrode buffer and Tris-citrate (5 mM citric acid, 30 mM Tris base) pH 8.5 as the gel buffer. Gels were run at 50 mA constant current for 2 h at 4 °C. GDH activity was detected by staining with a mixture of 10 mg/ml nitroblue tetrazolium, 2 mM NAD, 50 mM L-glutamate and 5 mM ADP in 30 ml followed by the addition after 1 h of 3 ml of 2 mg/ml phenazine methosulfate.

Chromatography

Chromatofocusing (methodology and reagents from Pharmacia Fine Chemicals) was carried out by layering enzyme preparation onto columns (0.7 × 50 cm) of PBE 94 exchanger pre-equilibrated in 25 mM imidazole buffer, pH 7.4 containing 10 mM 2-mercaptoethanol. Columns were eluted at 30 ml/h using 200 ml of Polybuffer 74 (a 1:8 dilution of stock) adjusted to pH 3.5. One ml fractions were collected and assayed for GDH activity.

For NAD-agarose chromatography tissue samples were homogenized in 10 mM imidazole-HCl buffer, pH 6.25 using a Polytron PT10-35 homogenizer at full speed for 3 five second bursts. Centrifugation was as above. Samples of supernatant were layered onto an NAD-agarose column (1 × 4 cm) equilibrated in the above buffer. The column was washed with buffer and then GDH was eluted using a 0 to 1 M gradient of KCl in buffer run at 30 ml/h. One ml fractions were collected and assayed for enzyme activity.

Enzyme Assay and Kinetic Study

GDH activity was monitored by following NAD(P)(H) utilization at 340 nm using a Gilford recording spectrophotometer with water jacketed cell holder for temperature control. Optimal assay conditions for either tissue were: 50 mM Tris-HCl buffer, pH 8.0, 50 mM L-glutamate, 2 mM NAD and 5 mM ADP in the glutamate oxidizing direction and 50 mM imidazole-HCl buffer, pH 7.5, 450 mM NH_4Cl, 10 mM α-ketoglutarate and 0.15 mM NADH for the NADH linked activity or 50 mM imidazole-HCl buffer, pH 6.75, 225 mM NH_4Cl, 10 mM α-ketoglutarate and 0.15 mM NADPH for the NADPH linked activity in the glutamate synthesizing direction. In the presence of 0.4 mM ADP optimal NH_4Cl concentration dropped to 225 and 135 mM for the NADH and NADPH linked activities, respectively. 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_H were determined from Hill plots using experimentally measured V_{max} values determined at saturating substrate concentrations. I_{50} values, the inhibitor concentration producing a 50% decrease in enzyme rate at saturating substrate concentrations, were determined from plots V (uninhibited)/V (inhibited) *versus* inhibitor concentration as outlined by Job et al. (1978). Apparent K_a values, the activator concentration producing a 50% increase in enzyme rate, were determined at subsaturating ammonium ion concentrations, 90 mM for the NADPH linked reaction and 150 mM for the NADH linked reaction.

Results

Enzyme Activity

The maximal activity (assayed in the absence of ADP) of GDH in *P. japonica* was 3.1 ± 0.5 U/g wet weight ($n=10$) in abdomen (fat body) and 24.8 ± 4.1 U/g wet weight ($n=10$) in thorax (flight muscle).

Physical Properties

Starch gel electrophoresis of fat body and flight muscle extracts showed differential migration of GDH from the two tissues. R_f values were 0.13 ± 0.01 ($n=3$) for flight muscle GDH and 0.35 ± 0.01 ($n=3$) for the fat body enzyme.

Both fat body and flight muscle GDH were highly unstable to chromatofocusing. An approximate p*I* of 5.3 ± 0.1 ($n=3$, 10% yield from column) was determined for fat body GDH but no accurate result was determined for the flight muscle enzyme.

At pH 6.25 both enzymes bound to NAD-agarose. When applied to the column separately and eluted with a gradient of KCl, fat body GDH eluted at 55 mM KCl while flight muscle GDH

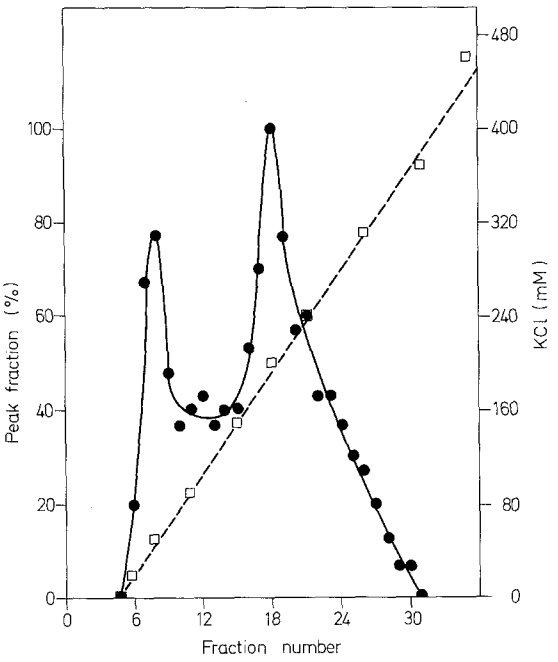

Fig. 1. Separation of *P. japonica* fat body and flight muscle GDH isozymes on NAD-agarose. Isozymes of GDH were bound to NAD-agarose as outlined in Materials and Methods and then eluted with a 0 to 1 M KCl gradient. Symbols are: ●, GDH activity; and □, KCl concentration

was retained until 170 mM KCl. When the two enzymes were mixed and applied to the column simultaneously similar elution profiles were seen, the fat body enzyme eluting at 50 mM and the flight muscle enzyme at 195 mM KCl (Fig. 1).

pH Optima and Coenzyme Utilization

GDH from both tissues showed the same pH optima, pH 8.5 for the forward direction and pH 7.5 and 6.75 for the NADH and NADPH linked reactions, respectively,of the reverse reaction. The acidic shift in pH optimum in the presence of NADPH versus NADH has been previously reported for other insect GDH's (Bond and Sang 1968; Male and Storey 1982). Under optimal substrate and pH conditions the ratio of NADH/NADPH activity was 6:1 for flight muscle and 8:1 for fat body. The relatively high NADPH activity of the flight muscle enzyme is contrary to the findings of Bursell (1975) who reported an NADPH activity of less than 2% of the NADH activity for tsetse fly flight muscle GDH. However this low percentage may have resulted if the differing pH optima and the differing optimal NH_4^+ concentrations (NADPH activity is strongly inhibited at NH_4^+ levels optimal for NADH activity) for the two coenzymes are not taken into account. For example, the ratio of NADH/NADPH activity was 18:1 at pH 7.5 but dropped to 2:1 at pH 6.75 while only 40% of maximal NADPH activity is detected when assays are run at NH_4^+ concentrations optimal for the NADH linked activity.

The ratio of NAD/NADP activities in the forward direction was 20:1 for flight muscle. The ratio of enzyme activity in the forward to reverse directions (NAD/NADH, at the respective pH optima) was 1:5 for fat body and 1:3.5 for flight muscle GDH.

The Reverse Reaction (Glutamate Synthesis)

A. Substrate Affinities. Table 1 shows the substrate affinities ($S_{0.5}$) and Hill coefficients (n_H) for the NADH and NADPH linked reactions of fat body and flight muscle GDH's. Affinity for α-ketoglutarate was significantly higher for the fat body enzyme, $S_{0.5}$ being 3 and 8.8 fold lower for the NADH and NADPH linked reactions, respectively. While flight muscle GDH showed no difference in affinity for α-ketoglutarate between the NADH and NADPH linked reactions, the NADPH linked

Table 1. Kinetic constants for the NADH and NADPH linked reactions of glutamate dehydrogenase from fat body and flight muscle of *P. japonica*

Tissue	Coenzyme	Substrate							
		α-Ketoglutarate		NAD(P)H		NH_4Cl		NH_4Cl (+0.4 mM ADP)	
		$S_{0.5}$	n_H	$S_{0.5}$	n_H	$S_{0.5}$	n_H	$S_{0.5}$	n_H
Fat body	NADH	0.81±0.09	1.3±0.1	41±2	1.4±0.1	170±12	6.3±1.0	79±13	3.0±0.5
	NADPH	0.26±0.03	1.3±0.2	46±2	1.7±0.4	99±12	1.9±0.2	34±3	2.3±0.3
Flight muscle	NADH	2.4 ±0.03	1.7±0.1	38±4	1.8±0.1	154±14	3.8±0.5	66±7	2.3±0.2
	NADPH	2.3 ±0.4	1.5±0.2	42±4	1.5±0.2	109±14	6.1±1.0	25±4	1.8±0.2

Results are means±s.e.m. for determinations on at least 3 separate enzyme preparations. $S_{0.5}$ values are in μM for NAD(P)H and in mM for α-ketoglutarate and NH_4Cl. Kinetic constants were derived at saturating cosubstrate concentrations as given in Materials and Methods with assays performed at pH 7.5 or 6.75 for the NADH or NADPH linked reactions, respectively

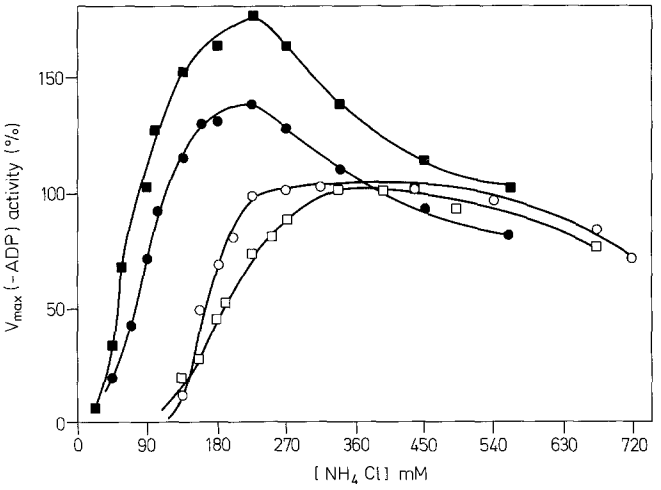

Fig. 2. Effect of the activator ADP (0.4 mM) on ammonium ion kinetics of the NADH-lined reaction of *P. japonica* fat body and flight muscle GDH. Assay conditions were as described in Materials and Methods. Symbols are: □, flight muscle (-ADP); ■, flight muscle (+ADP); ○, fat body (−ADP); and ●, fat body (+ADP)

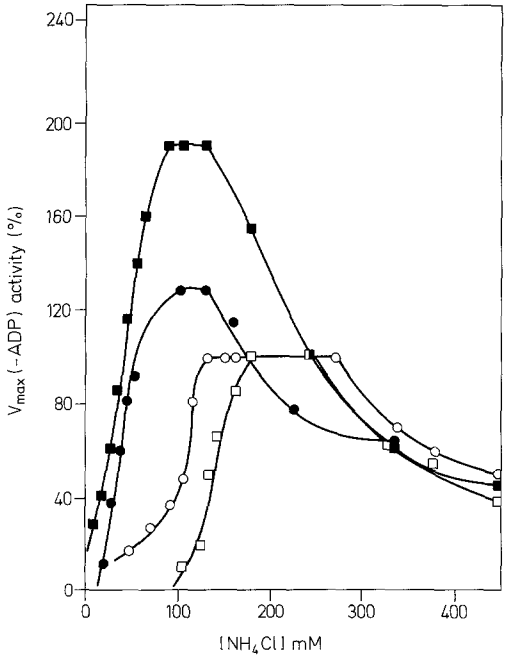

Fig. 3. Effects of the activator ADP (0.4 mM) on ammonium ion kinetics of the NADPH-linked reaction of *P. japonica* fat body and flight muscle GDH. Assay conditions were as described in Materials and Methods. Symbols are: □, flight muscle (−ADP); ■, flight muscle (+ADP); ○, fat body (−ADP); and ●, fat body (+ADP)

reaction of fat body GDH had a three fold greater affinity ($S_{0.5}$ three fold lower) for the keto acid compared to the NADH linked reaction. The two enzymes showed similar affinities for both NADH and NADPH. Ammonium ion kinetics were strongly sigmoidal for both enzymes and the NH_4^+ affinities of the two enzymes were similar for both the NADH and NADPH linked reactions. However both enzymes showed a significantly higher affinity for NH_4^+ in the presence of NADPH as the cofactor.

Table 2. Activator constants, $K_{a(app)}$, for ADP for glutamate dehydrogenase from fat body and flight muscle of *P. japonica*

Tissue	Coenzyme linked reaction	$K_{a(app)}$ mM
Fat body	NADH	0.067±0.010
	NADPH	0.034±0.002
	NAD	1.8 ±0.2
Flight muscle	NADH	0.038±0.004
	NADPH	0.016±0.005
	NAD	0.65 ±0.09

Apparent K_a values are means±s.e.m. for 3 determinations on separate enzyme preparations. $K_{a(app)}$ were determined at subsaturating NH_4Cl concentrations, 150 mM for the NADH linked reaction and 90 mM for the NADPH linked reaction with 0.15 mM coenzyme and 10 mM α-ketoglutarate. For the NAD linked reaction, substrate concentrations were 50 mM L-glutamate and 2 mM NAD

B. ADP Effects. ADP activated both fat body and flight muscle GDH. Maximal activation was 150% for the fat body enzyme and 180% for flight muscle GDH at 0.4 mM ADP, both NADH and NADPH linked reactions showing the same percentage activation by ADP. ADP affected ammonium ion kinetics only; Figs. 2 and 3 show the effects of ADP (at 0.4 mM) on the NADH and NADPH linked reactions, respectively. ADP lowered $S_{0.5}$ for NH_4^+ by 2 to 4 fold (Table 1), the decrease in $S_{0.5}$ being somewhat greater for the flight muscle enzyme than for fat body GDH. The effects of ADP concentration (at constant subsaturating NH_4^+ levels) are shown in Fig. 4 with apparent activation constants, K_a, given in Table 2. Apparent K_a for ADP was approximately two fold lower for the flight muscle enzyme indicating a tighter control of this enzyme by the activator.

C. Inhibitor Constants. Like GDH from other sources, GTP was an inhibitor of enzyme activity in both flight muscle and fat body while ATP inhibited the NADH linked reactions of both enzymes (Table 3). GTP was a strong inhibitor of flight muscle GDH with stronger inhibition (I_{50} four fold lower) of the NADPH linked reaction compared to the NADH reaction. However, GTP only weakly inhibited fat body GDH. ATP inhibi-

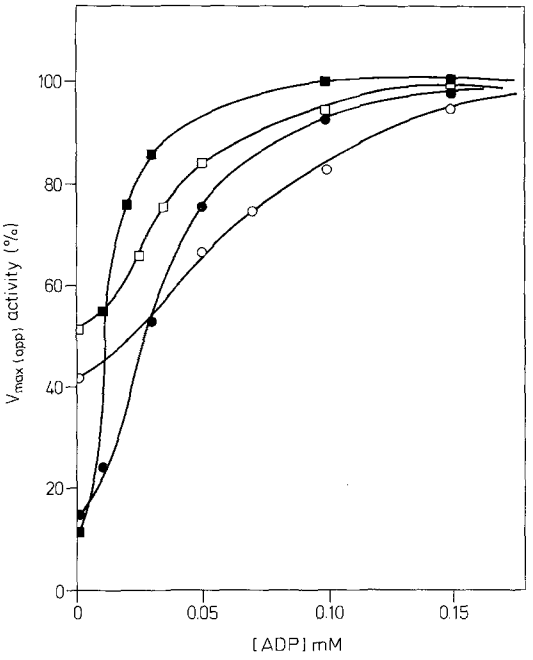

Fig. 4. Effects of varying the activator ADP at constant and subsaturating concentrations of ammonium ion (150 mM for the NADH and 90 mM for the NADPH linked reaction) on the reverse reaction of *P. japonica* fat body and flight muscle GDH isozymes. Assay conditions were as outlined in Materials and Methods. Symbols are: □, flight muscle (NADH); ■, flight muscle (NADPH); ○, fat body (NADH); and ●, fat body (NADPH)

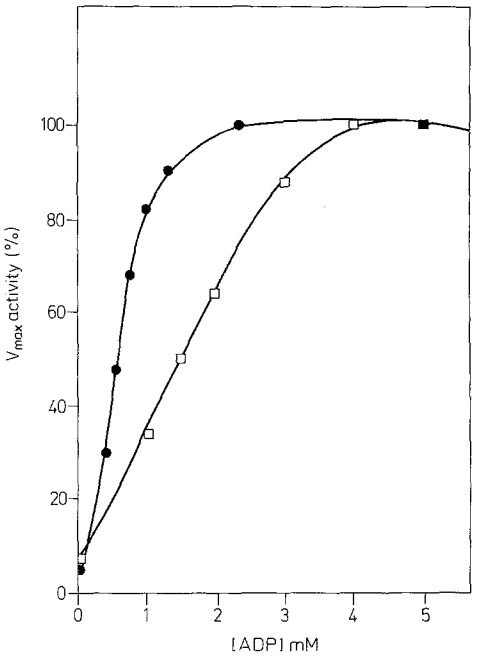

Fig. 5. Effects of varying the activator ADP on the NAD-linked forward reaction of *P. japonica* fat body and flight muscle GDH isozymes. Assay conditions were 50 mM L-glutamate and 2 mM NAD. Symbols are: □, fat body; and ●, flight muscle

Table 3. Inhibitor constants (I_{50}) for ATP and GTP for glutamate dehydrogenase from fat body and flight muscle of *P. japonica*

Tissue	Coenzyme linked reaction	I_{50} (ATP) (mM)	I_{50} (GTP) (μM)
Fat body	NADH	0.70±0.13	>500
	NADPH	–	>500
	NAD	0.29±0.06	60±6
Flight muscle	NADH	1.7 ±0.3	15.5±3.0
	NADPH	–	4.0±0.9
	NAD	0.48±0.11	6.5±0.9

I_{50} values are means±s.e.m. for 3 determinations on separate enzyme preparations. I_{50}'s were determined as outlined in Materials and Methods using saturating substrate concentrations. ATP is not an inhibitor of the NADPH linked reverse reaction

tion of the NADH linked reaction was slightly stronger for the fat body enzyme. Leucine, an inhibitor of mammalian GDH, did not affect either the NADH or the NADPH linked reactions of either enzyme from *P. japonica*.

The Forward Reaction (Glutamate Oxidation)

A. Substrate Affinities and ADP Effects. Activity of *P. japonica* GDH in the forward direction was strongly dependent upon the presence of ADP as an activator as is GDH from various other invertebrate sources (Storey et al. 1978; Male and Storey 1982). Enzyme activity increased by more than 10 fold in the presence of ADP (Fig. 5). Flight muscle GDH was more sensitive to ADP activation than was the fat body enzyme, the apparent K_a for ADP being three fold lower for flight muscle GDH (Table 2). Kinetic constants for the forward direction were determined in the presence of ADP (5 mM) and were similar in both tissues. $S_{0.5}$ for L-glutamate was 3.0±0.8 and 3.8±0.8 ($n=3$) for fat body and flight muscle GDH while $S_{0.5}$ for NAD was 0.22±0.02 and 0.17±0.03 ($n=3$) for the two tissues, respectively. Kinetics for both substrates were hyperbolic.

B. Inhibitor Constants. GTP inhibition of flight muscle GDH was strong with an I_{50} of 6.5 μM (Table 3). Fat body GDH, however, was only weakly inhibited by GTP. ATP inhibition of the forward reaction was stronger with respect to fat body GDH.

Discussion

Tissue specific forms of glutamate dehydrogenase in *P. japonica* are distinguishable by both physical and kinetic differences. This is the first demonstra-

tion of the occurrence of isozymic forms of GDH amongst animal species. Studies in mammalian systems have been fairly conclusive in showing the absence of isozymic forms of this key enzyme amongst higher vertebrates (Frieden 1965) but the occurrence of GDH isozymes amongst lower vertebrates and throughout the invertebrate phyla should now be closely examined.

Flight muscle and fat body GDH from the Japanese beetle differ strongly in their kinetic properties. The flight muscle enzyme is much more strongly regulated by nucleotides showing a lower apparent K_a value for ADP and stronger inhibition by GTP. Although inhibition by ATP appears to be slightly weaker for the flight muscle enzyme than for fat body GDH, total adenylate pools in flight muscles are typically much higher (6 μmol/g wet weight) than in soft tissues of insects (2 μmol/g)(Sacktor and Hurlbert 1966; Mandel et al. 1980; K.B. Storey, unpublished results). Indeed, Hansford and Johnson (1975) demonstrated that glutamate oxidation by *P. japonica* flight muscle GDH is highly sensitive to adenylate energy charge. In their studies enzyme rate was maximal at an energy charge of 0.5 to 0.7 (total adenylates = 5 mM), declined sharply at an energy charge greater than 0.90 and was not detectable at values above 0.98. Thus the oxidation of glutamate (derived from proline) would be readily activated at the initiation of flight when ADP levels rise and ATP (and GTP) content falls. Alternately, high energy charge (via GTP inhibition) would turn off glutamate oxidation in resting muscle. Reverse functioning of the flight muscle isozyme in amino acid biosynthesis might be strongly limited in vivo by the very high $S_{0.5}$ for α-ketoglutarate and the strong GTP inhibition of the enzyme.

Fat body GDH, on the other hand, shows only limited control by nucleotides. ADP activation of the enzyme is lower and GTP inhibition is minimal particularly in the direction of glutamate synthesis. The activity of fat body GDH, therefore, is not tightly linked to the energy status of the cell with the consequence that the enzyme could function well under high cellular energy states. Regulation of fat body GDH for a biosynthetic role is probably tied to substrate availability, the enzyme displaying a particularly high affinity for α-ketoglutarate in the presence of NADPH as the cofactor. Utilizing α-ketoglutarate produced from carbohydrate reserves and recycling NH_4^+ released during proline oxidation in working flight muscle, fat body GDH would have a key role in the biosynthesis of amino acids and the restoration of proline reserves in the insect.

While fat body GDH has an important biosynthetic function, the flight muscle enzyme is linked to the oxidation of proline as a flight fuel. Mitochondria from *P. japonica* flight muscle oxidize proline, pyruvate and glycerol-3-phosphate (Hansford and Johnson 1975). Oxidation of proline leads to the production of greater amounts of ammonia than of alanine demonstrating that the catabolism of glutamate proceeds largely via the action of GDH rather than through transamination reactions. This differs from the route of proline oxidation in some other species (tsetse fly, *Glossina morsitans;* Colorado potato beetle, *Leptinotarsa decemlineata*) which accumulate alanine as the product of proline catabolism (Bursell and Slack 1976; Weeda et al. 1980). In *P. japonica* the carbon skeleton of proline is introduced to the Krebs cycle via GDH. α-Ketoglutarate is catabolized to malate and malate is converted to pyruvate via a high activity malic enzyme. Pyruvate is then oxidized by the Krebs cycle. The overall result is a complete oxidation of proline producing NH_3, CO_2 and H_2O (Hansford and Johnson 1975). The properties of flight muscle GDH are consistent with this function; nucleotide effects (ADP activation, release of ATP/GTP inhibitions) as well as rising glutamate levels during the oxidation of proline would activate the oxidation of glutamate via GDH during the initiation of flight.

Glutamate dehydrogenase in mammalian systems apparently catalyzes both catabolic and anabolic functions both within a single cell and between different tissue types. Regulation of these is believed to result from the complex regulatory controls of the enzyme including the modulating effects of nucleotides (Goldin and Frieden 1971; Smith et al. 1975). Nucleotide effects (GTP inhibition, ADP activation), however, seem to be at odds with the biosynthetic function of the enzyme; biosynthesis should occur when cellular energy reserves are high, i.e. when GTP levels are high and ADP levels low. A clue to the functional origin of the regulatory effectors of GDH could be taken from this study. Flight muscle GDH, with its catabolic function, is tightly regulated by nucleotides. The anabolic fat body GDH shows only limited regulation by nucleotides. For the biosynthetic function of GDH in the fat body, substrate availability and the lack of ATP inhibition of the NADPH linked reaction may be the important regulatory factors. By analogy we could suggest that the nucleotide regulation typical of GDH from all higher vertebrate sources has its origin in the control of the glutamate oxidizing activity of the enzyme. The glutamate synthesizing activity of the

enzyme, although affected by nucleotides in vitro, may be less responsive to nucleotide control in vivo with control of enzyme activity largely vested in substrate availability instead.

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