Influence of L-Leucine on Glutamate Dehydrogenase Activity in Isolated Rat Diaphragm

GEORGE PALAIIOLOGOS AND PHILIP FELIG

Yale University School of Medicine, New Haven, Connecticut

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Glutamate dehydrogenase (NAD) activity was measured in liver and diaphragm mitochondria from 48 h fasted rats. Kinetic studies were performed with diaphragm enzyme and the effects of L-leu, ADP and L-ala on the K'm and V'max for NH₄⁺, and α-ketoglutarate were evaluated. L-leucine increases by 2–8 fold the V'max for all three substrates with no significant changes in the K'm. ADP increased by 3–7 fold the V'max for all three substrates and the K'm for NADH and α-ketoglutarate by 1.5–7.0 fold. L-alanine had no effect on either the V'max or the K'm of any substrate. The results suggest that muscle has the capacity to form glutamate through the glutamate dehydrogenase reaction and that L-leucine may stimulate this reaction in muscle.

Alanine is released by the human myocardium [1] and skeletal muscle [2] in amounts exceeding that derived from protein catabolism. These observations led to the suggestion that alanine is synthesized de novo in muscle from pyruvate and glutamate through the alanine aminotransferase reaction [3]. Similar results were obtained with the perfused rat hindquarter [4] and the incubated rat diaphragm [5, 6]. Glutamate for alanine production in muscle has been suggested to be derived from the branched-chain amino acids [7] and it has been demonstrated in the isolated rat diaphragm that branched-chain amino acids increase alanine production [5, 6]. Increased production of alanine has also been observed in exercise [8, 9] a condition in which ammonia production in muscle is increased [10] via the purine nucleotide cycle [11]. In addition, blood alanine concentration is elevated in a number of cases where hyperammonaemia is noted [12–15]. On the basis of these observations it has been suggested that alanine serves as a carrier of nitrogen in cases of increased formation or inadequate disposal of ammonia [16]. Whether muscle tissue has the capacity to synthesize glutamate from ammonia and thereby provide ammonia-derived nitrogen for alanine formation has not, however, been established. The present study was consequently undertaken to investigate the activity and kinetics of glutamate dehydrogenase in rat diaphragm and to compare this enzyme with that found in liver tissue. Since leucine is involved in the production of alanine in muscle [5, 6] and in nitrogen metabolism in liver mitochondria [17], the effect of leucine on the kinetic parameters of rat diaphragm glutamate dehydrogenase has also been studied.

MATERIALS AND METHODS

Male Wistar rats were from Microbiological Associates and starved for 58 h. Water was given ad libitum. At the time of death the rats weighted 150–200 g.

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3Please address reprint requests to Philip Felig, M.D., Department of Internal Medicine, Yale School of Medicine, 333 Cedar Street, New Haven, Conn. 06510
0044-0086/78/5101-0019 $00.80
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FIG. 1. Kinetics of the glutamate dehydrogenase reaction (NAD) with NH$_4^+$ as the variable substrate from rat diaphragm mitochondria.

Rat diaphragm mitochondria were isolated and glutamate dehydrogenase was extracted as described in the Materials and Methods section. Initial velocities were determined as described in Table 1. The constant concentrations of NADH and α-ketoglutarate were 0.22 mM and 3 mM respectively. The effectors used were control (○), 1 mM leu (□), 3 mM leu (Δ) and 5 mM leu (A) (Fig. 1A) and control (○), 0.5 mM ADP (♦), 1 mM ADP (■), 2 mM ADP (▲) and 1-5 mM ala (♦) (Fig. 1B).

Trypsin (236 U/mg) was from Worthington Biochemical Corp. Lubrol WX, albumin, adenosine 5-diphosphate, L-alanine, and triethanolamine hydrochloride were from Sigma. NADH (disodium salt; grade II) and α-ketoglutaric acid were from Boehringer (Mannheim). L-leucine was from Schwartz/Mann. All other chemicals were from Mallinckrodt and were of the highest purity available.

Beginning at 9:00 A.M. each of twenty animals was decapitated and the liver and diaphragm were removed quickly and placed in separate beakers containing ice-cold 0.14 M-sucrose containing 50 mM-potassium phosphate buffer, pH 7.2 [18]. The tissues were chopped lightly with scissors and washed free of blood by twice decanting the liquid. Diaphragms, 5–6 g, were incubated for 30 min with constant stirring in 40 ml of sucrose-phosphate buffer containing 5–6 mg trypsin at ice-bath temperature [19]. The suspension was centrifuged at 110000 g-min; the precipitate was suspended in the sucrose-phosphate buffer (1:9) and homogenized in a Dounce homogenizer by hand. Livers were homogenized in the same buffer (1:9)
FIG. 2. Kinetics of the glutamate dehydrogenase reaction (NAD) with NADH as the variable substrate from rat diaphragm mitochondria.

Rat diaphragm mitochondria were isolated and glutamate dehydrogenase was extracted as described in the Materials and Methods section. Initial velocities were determined as described in Table 1. The constant concentrations of NH4+ and α-ketoglutarate were 40 mM and 3 mM respectively. Notations for the effectors are shown in Fig. 1.

with a Dounce homogenizer. Both diaphragm and liver homogenates were centrifuged in a Sorval; RC2-B centrifuge as described by Chappel & Hansford [20]. The final mitochondrial pellet from the diaphragms and the livers were taken up with buffer. The diaphragm mitochondria were brought to 10 ml. In these suspensions protein determination was carried out by the method of Lowry et al. [21] as described by Bergmeyer et al. [22] with bovine serum albumin as standard. Both mitochondrial suspensions were diluted with buffer containing lubrol, 0.5 mg per mg of mitochondrial protein [23]. The final dilutions contained 2 mg/ml protein from liver mitochondria and 10 mg/ml protein from diaphragm mitochondria. The suspensions were mixed with a magnetic stirrer at ice-bath temperature for 15 min, centrifuged at 110,000 g-min and the supernatant was used for glutamate dehydrogenase determination.

The glutamate dehydrogenase activity was determined as described by Brdiczka et al. [24]. The assay medium was 330 mM in sucrose [25]. The volumes of mitochondrial suspension used for the determination of the enzyme activity ranged from 5 to 50 μl for the liver and from 10 to 100 μl for the diaphragm. Within this range there was proportionality between reaction velocity and enzyme concentration. The specific activity was expressed as μmoles of NADH oxidized per min per mg of mitochondrial protein at 25°C and pH 7.6. Initial velocities were determined in the absence and in the presence of L-leucine (1, 3 or mM); ADP (0.5, 1 or 2 mM) or L-alanine (1, 3 or 5 mM) varying the concentration of one substrate and keeping the
FIG. 3. Kinetics of the glutamate dehydrogenase reaction (NAD) with α-ketoglutarate as the variable substrate from rat diaphragm mitochondria.

Rat diaphragm mitochondria were isolated and glutamate dehydrogenase was extracted as described in the Materials and Methods section. Initial velocities were determined as described in Table 1. The constant concentrations of NH₄⁺ and NADH were 40 mM and 0.22 mM respectively. Notations for the effectors are shown in Fig. 1.

concentrations of the other two substrates constant. [NH₄⁺] ranged from 2.5 mM to 40 mM, [α-ketoglutarate] from 0.1 mM to 4.0 mM and [NADH] from 0.025 mM to 0.4 mM. The constant [NH₄⁺], [α-ketoglutarate] and [NADH] were 40 mM, 3 mM and 0.22 mM respectively. The apparent Km (Kᵡm) and apparent Vmax (Vᵡmax) for α-ketoglutarate, NADH and NH₄⁺ in the presence and the absence of the effectors were calculated by the Lineweaver-Burk plot. The straight lines were drawn by a computer program.

RESULTS

The specific activities of glutamate dehydrogenase in the presence of 2 mM ADP were determined for the diaphragm and liver mitochondria in six different preparations.

The mean values ±S.E.M. for the diaphragm and liver glutamate dehydrogenases were 0.120±0.016 and 1.80±0.20 μmoles/min/mg mitochondrial protein, respectively. The mean values ±S.E.M. for the diaphragm and liver mitochondrial protein isolated were 4.5±0.3 and 12.6±0.9 mg mitochondrial protein/g wet wt. tissue respectively.

Figures 1–3 show the plots of the initial velocities of the diaphragm enzyme against the concentrations of each substrate, keeping the concentrations of the
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mitochondria were isolated and the glutamate dehydrogenase extracted as described in the Material and Methods section. The supernatants (10–100 μl) after centrifugation of the lubrol treated mitochondria, were added in 3.0 ml 0.33 M-sucrose containing 50 mM triethanolamine, 5 mM EDTA, and varying concentrations of substrates (NH4+, NADH, and α-ketoglutarate) and effectors (leucine, ADP, and alanine) as described in the Materials and Methods section. The reaction was started by the addition of α-ketoglutarate. Blanks contained no α-ketoglutarate. The specific activity was expressed as μmoles NADH oxidized per min per mg of mitochondrial protein at 25°C and pH 7.6. K'm and V'max values were calculated by the Lineweaver-Burk plots of the points in Figs. 1–3.

TABLE 1

Ratios of K'm and V'max for diaphragm glutamate dehydrogenase

| Substrate | Effector | K'm with effector | V'max with effector |
|-----------|----------|------------------|-------------------|
| NH4+ (Control K'm = 15 mM) | Leucine | 1.1–1.5 | 3.0–8.0 |
| | ADP | 1.1–1.7 | 4.0–7.0 |
| | Alanine | 0.9–1.1 | 1.3–1.5 |
| NADH (Control K'm = mM) | Leucine | 1.2–1.8 | 2.5–3.5 |
| | ADP | 2.5–7.0 | 4.0–7.3 |
| | Alanine | 0.9–1.2 | 1.1 |
| α-ketoglutarate (control K'm = mM) | Leucine | 0.9–1.6 | 1.8–3.1 |
| | ADP | 1.4–3.0 | 2.6–2.9 |
| | Alanine | 0.9–1.0 | 1.1–1.4 |

The other two substrates constant, in the absence and in the presence of L-leucine, ADP or L-alanine. It is evident from these figures that the rat diaphragm enzyme, as the liver enzyme, is activated by L-leucine and by ADP and it is unaffected by L-alanine. To obtain a quantitative measure of the observed effects, the values of the activities were replotted as a double reciprocal plot and the obtained values for K'm and V'max in the presence and the absence of the effectors are shown in Table 1. Table 1 shows that L-leucine and ADP increase markedly (3–8 fold) the V'max for NH4+ and NADH and to a lesser extent (2–3 fold), the V'max for α-ketoglutarate. In addition, ADP increases the K'm for NADH and α-ketoglutarate to the same extent as the V'max. L-alanine has no significant effect on either V'max or K'm.

DISCUSSION

The present experiments show that the mean specific activity of glutamate dehydrogenase from rat diaphragm is 0.120 μmoles/min/mg mitochondrial protein. Previous studies examining this enzyme in diaphragm have not involved isolation of mitochondria and have expressed the specific activity per wet wt of tissue [26, 27].

Inasmuch as glutamate dehydrogenase and leucine aminotransferase provide alternative pathways for glutamate production and subsequent alanine synthesis, it is of interest to compare the activities of these enzymes in muscle. The reported values for the L-leucine aminotransferase in skeletal muscle are 0.25–0.56 μmole/min/g wet wt [28, 29]. Since 4.5 mg of mitochondrial protein were isolated from one g of wet diaphragm, the specific activity of glutamate dehydrogenase per g of wet tissue may be estimated as 0.54 μmoles/min/g wet wt. In view of the known loss of mitochondria during the isolation procedure [18] this value is highly underestimated
 particularly with muscle mitochondria of fasted rats. These results thus suggest that diaphragm has the capacity to form glutamate through the glutamate dehydrogenase reaction at rates at least comparable to the rate that glutamate is formed through the L-leucine aminotransferase reaction.

The present experiments also show that glutamate dehydrogenase from rat diaphragm is activated by L-leucine and ADP and is unaffected by L-alanine. Similar results have been reported for the liver enzyme of the ox and the rat [30, 17]. The stimulatory effects of L-leucine were demonstrable at concentrations as low as 1 mM and in the case of ADP at concentrations of 0.5 mM. Since muscle ADP has been reported to be almost totally bound to actin [31, 32], whether ADP can exert a physiological role in the regulation of glutamate dehydrogenase in muscle remains questionable. On the other hand, the concentration of free L-leucine in rat diaphragm is in the range of 0.2 mM in the fasting state [33] and probably rises substantially after protein feeding [34, 35].

If the current in vitro findings demonstrating a stimulatory effect of leucine on glutamate dehydrogenase apply to the in vivo condition, then leucine may have a dual role in alanine synthesis, functioning as substrate as well as enzyme activator (Fig. 4). Under conditions of increased leucine catabolism, [glutamate] will increase and [leucine] will decrease. Both events will affect glutamate dehydrogenase activity. The increase in [glutamate] will inhibit glutamate dehydrogenase [36] while the decrease in [leucine] will deactivate the enzyme. In this circumstance, glutamate for alanine production will be synthesized primarily from leucine. Under conditions of decreased catabolism of leucine, leucine will accumulate and activate glutamate dehydrogenase. In this situation, glutamate for alanine formation will come primarily through the glutamate dehydrogenase reaction at the expense of NH₄⁺. By this scheme, leucine may have a regulatory role in alanine synthesis in circumstances of increased as well as decreased leucine catabolism.

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LEUCINE AND GLUTAMATE DEHYDROGENASE

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