The effects of phospholipids on the activation of glutamate dehydrogenase by L-leucine

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Glutamate dehydrogenase in disrupted mitochondrial preparations is activated by L-leucine to a much greater extent than is the purified enzyme. A factor, or factors, responsible for modulating the sensitivity of L-leucine is lost during the purification of the enzyme. Although both cardiolipin and phosphatidylserine are inhibitors of the enzyme, only the inhibition by the former phospholipid is reversed by L-leucine. The inhibition of glutamate dehydrogenase by its binding to cardiolipin in the disrupted mitochondrial preparations and its relief by L-leucine could account for the greater sensitivity of such preparations to activation by that amino acid.

INTRODUCTION

L-Leucine is a poor substrate for glutamate dehydrogenase [L-glutamate:NAD(P)⁺ oxidoreductase (deaminating); EC 1.4.1.3] and a potent allosteric activator of the enzyme (see Syed & Engel, 1984). McGivan *et al.* (1973) reported that the extent of activation of glutamate dehydrogenase in intact or sonically disrupted mitochondrial preparations by L-leucine was significantly greater than had been reported for purified preparations of the enzyme (see Yielding & Tomkins, 1961). These results are, however, difficult to compare directly, since the sources of the enzyme preparations and the assay conditions were different in the two studies. The kinetic properties of the enzyme have been shown to be affected by different assay buffer conditions (Engel & Dalziel, 1969).

It has been shown that limited proteolytic cleavage can occur during the purification of glutamate dehydrogenase from ox liver and brain and that this results in the production of an enzyme species with modified kinetic and regulatory properties (McCarthy *et al.*, 1980, 1981; McCarthy & Tipton, 1985). Although preparations of the enzyme which had suffered proteolysis in this way differed from the native enzyme in their responses to L-leucine under some assay conditions, it appeared that these changes were not large enough to account for the reported differences between the enzyme in mitochondrial and purified preparations (Couée & Tipton, 1989).

A factor which might affect the activity of glutamate dehydrogenase in mitochondrial preparations is its interaction with phospholipids. Several studies have shown that phospholipids, such as cardiolipin and phosphatidylserine, bind to the enzyme and inhibit its activity (Julliard & Gautheron, 1972; Dodd, 1973; Godinot, 1973; Nemat-Gorgani & Dodd, 1977a,b; Pour-Rahimi & Nemat-Gorgani, 1987). The purpose of the present studies was to compare the activation of glutamate dehydrogenase in disrupted mitochondria and purified preparations by L-leucine under the assay conditions used by McGivan *et al.* (1973) and to investigate the possibility that interactions with phospholipids might

modulate these responses. A preliminary report of part of this work has been published (Couée & Tipton, 1987).

MATERIALS AND METHODS

Enzyme preparations

Ox brain and liver glutamate dehydrogenase were purified to apparent homogeneity and shown not to have suffered significant proteolytic cleavage by the procedures previously described (McCarthy *et al.*, 1980). An apparently homogeneous preparation of the enzyme from rat brain was obtained by the procedure of Graham *et al.* (1985).

Mitochondria were prepared from freshly excised livers of rats (Wistar) which had been fasted overnight, or from ox livers obtained from the abattoir as soon as possible after death, by a procedure based on that of Kearney *et al.* (1971). All operations were carried out at 0–4 °C. The livers were washed with 3 vol. (v/w) of 10 mM-Tris/HCl buffer, pH 7.2, containing 0.25 M-sucrose, blotted dry, cut thinly and homogenized in 3 vol. of the same buffer in a Thomas homogenizer by using three passes of a 0.65 mm (0.026 in)-clearance pestle, followed by three passes of a 0.30 mm (0.012 in)-clearance pestle. The homogenate was centrifuged at 800 g for 15 min and the supernatant was retained. The pellet was resuspended in 2 vol. of the same buffer mixture and centrifuged as above. The combined supernatants were centrifuged at 8500 g for 15 min and the pellet obtained was washed by suspension and centrifugation in about 2 vol. of the buffer mixture. The resultant mitochondrial pellet was suspended in 0.1 M-phosphate buffer, pH 7.2, to give a protein concentration of at least 20 mg/ml, and stored at –20 °C until required. After thawing, samples (about 0.5 ml) were disrupted by sonication in a Mettler Cavitator bath for three 1 min periods, with intervening times of at least 5 min on ice.

A rapid partial purification was carried out by a procedure adapted from earlier stages of that of McCarthy *et al.* (1980). All steps were carried out at 0–4 °C. A 0.4 ml portion of the sonicated mitochondrial

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suspension (at least 20 mg of protein/ml) was adjusted to 20 % satn. with $(\text{NH}_4)_2\text{SO}_4$ and shaken gently for 20 min before centrifugation at 18000 *g* for 16 min. The supernatant was adjusted to 50 % satn. with $(\text{NH}_4)_2\text{SO}_4$, shaken for 20 min and centrifuged as above. The pellet obtained was suspended in 0.4 ml of 20 mM-phosphate buffer, pH 7.4. About 0.1 ml of this sample was applied to a 6 cm $\times$ 1 cm column of Sephadex G-75 equilibrated and eluted with the same buffer; 0.5 ml fractions were collected, and those containing enzyme activity were pooled and applied to a 1.5 cm $\times$ 0.5 cm column of DEAE-cellulose (Whatman DE-52) which had been equilibrated with 20 mM-phosphate buffer, pH 7.2. The column was washed with 10 vol. of the same buffer, and glutamate dehydrogenase was then eluted with 200 mM-phosphate buffer, pH 7.4. The entire procedure could be carried out in 1 day.

Enzyme assay

Enzyme activities were determined spectrophotometrically by monitoring the oxidation of NADH at 340 nm. Highly purified preparations of the enzyme were added to the assay mixtures to give concentrations in the range 0.04–0.1 $\mu\text{g/ml}$. Ultrasonically disrupted mitochondria were added to assay mixtures to give protein concentrations in the range 20–200 $\mu\text{g/ml}$. Partially purified enzyme preparations from that source were added to give comparable velocities (0.4–2 nmol/min) in the absence of L-leucine.

Unless otherwise stated, assays were carried out at 25 °C in a medium containing, in a total volume of 2.5 ml, 0.125 M-KCl, 0.025 M-Tris/HCl, pH 7.3, 20 mM- NH_4Cl , 160 μM -NADH, the enzyme sample and 2.5 mM-2-oxoglutarate, which was added to start the reaction. These assay conditions were those used by McGivan *et al.* (1973) in their studies on the activation of the mitochondrial enzyme. In studies where the activities of mitochondrial preparations or partially purified glutamate dehydrogenase preparations were compared with the purified enzyme, the assay mixture also contained 0.4 mM-KCN and, where indicated, 0.4 % (v/v) Triton X-100.

In the studies on the effects of phospholipids dissolved in ethanol, the assays were carried out as described by Dodd & Radda (1969) at 25 °C in a medium containing, in a final volume of 2.5 ml: 20 mM-Pipes, 20 mM-Hepes, 20 mM-Tris, pH 7.2, 50 mM- NH_4Cl , 100 μM -NADH and 5 mM-2-oxoglutarate.

Phospholipid preparations

Cardiolipin (ox heart), phosphatidylcholine (L- α -dimyristoylphosphatidylcholine) and phosphatidylserine (ox brain) were obtained from Sigma. Determination of the absorbance spectra of solutions in ethanol revealed no absorbance peak at 233 nm, indicating that no oxidized phospholipid derivatives were present (Klein, 1970). The phospholipids were recovered from ethanol by evaporation under N_2 and dispersed by sonication in a medium containing 0.02 M-Hepes, 0.02 M-Pipes and 0.02 M-Tris, pH 7.7 (Nemat-Gorgani & Dodd, 1977a). The medium was flushed with N_2 for at least 10 min before use. Sonication was carried out at 0–4 °C under N_2 in a Mettler Cavitator bath for at least three 1 min periods with intervals of at least 5 min on ice. Phosphatidylcholine and phosphatidylserine samples (900 μg)

were suspended in 1 ml of sonication medium, and 250–750 μg samples of cardiolipin were suspended in 0.4–2.0 ml of medium. Phospholipid dispersions were kept at 0–4 °C, in rubber-stoppered vials under N_2 throughout each experiment, with samples being removed through the stopper with a microlitre syringe.

Other assay methods

Protein concentrations were determined either by the method of Markwell *et al.* (1978) or, for more highly purified preparations, by measuring the absorbance at 280 nm. A 1 mg/ml solution of glutamate dehydrogenase was assumed to give an absorbance at this wavelength of 0.97 cm^{-1} (Olson & Anfinsen, 1952).

Phospholipids were extracted from pure glutamate dehydrogenase samples by the method of Folch *et al.* (1951) and determined by their phosphate content by the method of Raheja *et al.* (1973).

RESULTS

Purified ox liver and brain glutamate dehydrogenase responded identically to activation by L-leucine under the conditions used in these studies. However, the activity in the mitochondrial preparations, whether disrupted by sonication or by treatment with Triton X-100, was stimulated to a significantly greater extent by this amino acid (Table 1). There was some variability in the degree of activation by L-leucine. However, alternate comparisons of different preparations gave consistent results. Commercially obtained ox liver glutamate dehydrogenase (from Boehringer) was less sensitive to activation than were the laboratory-purified ox liver and brain preparations when assayed at 160 μM -, but not at 40 μM -, NADH (Couée & Tipton, 1989).

The rapid purification procedure gave a final purification of ox liver glutamate dehydrogenase of some 25-fold, with a yield of 37 % (Table 2). Monitoring the activation by L-leucine at the different stages of the purification showed that, whether assayed in the absence or presence of 0.4 % Triton X-100, the degree of activation declined until it was not significantly different from that observed with the apparently homogeneous preparations from ox brain (Table 3).

Table 1. Activation of glutamate dehydrogenase preparations by L-leucine

Initial velocities were measured in the absence (v_0) and presence (v) of L-leucine. In the Triton-treated samples the assay mixture also contained 0.4 % (v/v) Triton X-100. The numbers of determinations are shown in parentheses. Results are mean values $\pm$ S.E.M., except for the purified ox brain enzyme in the presence of Triton X-100, when it is the mean value $\pm$ range.

Preparation	Treatment	$(v - v_0)/v_0$
Rat liver mitochondria	Sonication	9.5 ± 0.8 (17)
	Triton	11.0 ± 1.0 (6)
Ox liver mitochondria	Sonication	11.0 ± 0.8 (14)
	Triton	8.5 ± 0.3 (14)
Purified ox brain	None	3.5 ± 0.2 (19)
	Triton	2.7 ± 0.6 (2)

Table 2. Rapid partial purification of glutamate dehydrogenase from ox liver mitochondria

The purification procedure was as described in the text. A unit of enzyme activity is defined as that catalysing the oxidation of 1 μ mol of NADH in 1 min under the assay conditions described in the text. Protein concentration was determined by the method of Markwell *et al.* (1978) at each step, except for the final stage, when the A_{280} was used.

Procedure	Volume (ml)	Protein concn. (mg/ml)	Activity (unit/ml)	Specific activity (unit/mg)	Purification factor	Yield (%)
Sonicated mitochondria	0.4	25	0.75	0.03	1	100
(NH ₄) ₂ SO ₄ pellet	0.4	2.5	0.5	0.2	7	67
Gel-filtration eluate	6	0.1	0.024	0.24	8	48
Ion-exchange eluate	2	0.055	0.04	0.7	25	37

Table 3. Activation of glutamate dehydrogenase preparations obtained during its partial purification from ox liver mitochondria by L-leucine in the absence and presence of 0.4% (v/v) Triton X-100

v and v_0 represent the activities determined in the presence and absence, respectively, of 5 mM-L-leucine. The purification procedure was as described in the text. Results are mean values $\pm$ S.E.M., or range when duplicate determinations only were carried out, and the numbers of determinations are given in parentheses.

Preparation	$(v - v_0)/v_0$	
	No Triton	Plus Triton
Sonicated mitochondria	11.0 $\pm$ 0.8 (14)	8.5 $\pm$ 0.3 (14)
(NH ₄) ₂ SO ₄ pellet	10.0 $\pm$ 0.5 (2)	8.0 $\pm$ 0.1 (6)
Gel-filtration eluate	6.0 $\pm$ 0.5 (9)	7.6 $\pm$ 0.6 (4)
Ion-exchange eluate	2.7 $\pm$ 0.5 (5)	3.4 $\pm$ 0.2 (3)
Purified ox brain	3.5 $\pm$ 0.2 (19)	2.7 $\pm$ 0.6 (2)

The effects of phospholipids on the activity of purified glutamate dehydrogenase were found to be constant over the time period of the experiments. Rates of the enzyme-catalysed reaction were linear in the presence as well as the absence of the phospholipids, indicating that no time-dependent effects were occurring. When the effects of phosphatidylcholine and phosphatidylserine dissolved in ethanol, in accordance with the procedure of Julliard & Gautheron (1972), were studied under the assay conditions of Dodd & Radda (1969), it was found that ethanol alone inhibited the enzyme at concentrations higher than 90 mM. Phosphatidylcholine was found to have no effect on the activity of the enzyme at concentrations up to 80 μ g/ml (120 μ M). In contrast, as shown in Fig. 1, phosphatidylserine was a potent inhibitor of glutamate dehydrogenase. Because ethanol might affect the interactions of glutamate dehydrogenase with other effectors, subsequent experiments were performed with phospholipid suspensions prepared by sonication (Dodd, 1973).

The inhibition of glutamate dehydrogenase by phosphatidylserine was not significantly affected by the presence of 0.4% (v/v) Triton X-100. It was, however, decreased by the presence of KCl, as might be expected

Fig. 1. Inhibition of ox brain glutamate dehydrogenase by phosphatidylserine

The enzyme preparation was assayed by the method of Dodd & Radda (1969) in the presence of increasing concentrations of phosphatidylserine. The phosphatidylserine was added from a stock solution of 0.088 mg/ml. The maximum amount of ethanol added to the assay was 8 μ l, and this had no effect on the activity of the enzyme when added alone. Each experimental point represents the mean of duplicate determinations.

from the results of Nemat-Gorgani & Dodd (1977a), who showed high ionic strengths to decrease the inhibition of the enzyme by phospholipids. Thus, as shown in Fig. 2, higher concentrations of phosphatidylserine were required for inhibition in the presence of 0.125 M-KCl than its absence (see Fig. 1).

Cardiolipin was also found to inhibit the activity of the enzyme under these conditions. The concentration-dependence of the inhibition of glutamate dehydrogenase by sonicated phospholipids was, however, variable. Thus the concentrations of phosphatidylserine and cardiolipin necessary to give 50% inhibition were found to vary in the ranges 15–70 μ g/ml and 2–40 μ g/ml respectively. Similar variability has been reported by others, with the concentrations of cardiolipin and phosphatidylserine

Fig. 2. Effects of phosphatidylserine on the activation of ox brain glutamate dehydrogenase by L-leucine

Phosphatidylserine dispersions were prepared by sonication and enzyme activity was determined, as described in the text, in the presence of different concentrations of L-leucine and the following fixed concentrations of phosphatidylserine; 0 (■), 36 $\mu\text{g/ml}$ (●) and 72 $\mu\text{g/ml}$ (▲). Each point represents the mean of triplicate initial-velocity determinations. Results are shown in the inset as plots of $(v - v_0)/v_0$ versus the L-leucine concentration, where v_0 and v represent the velocities in the absence and presence of L-leucine respectively.

Fig. 3. Effects of cardiolipin on the activation of ox brain glutamate dehydrogenase by L-leucine

The cardiolipin concentrations were: 0 (■), 2.6 $\mu\text{g/ml}$ (●) and 7.8 $\mu\text{g/ml}$ (▲). Other details were as described in Fig. 2. The inset shows the data plotted as described for Fig. 2 inset.

varying by factors of 100 and 10 respectively (Dodd, 1973; Nemat-Gorgani & Dodd, 1977a). Suspensions of phosphatidylcholine had no effect on the activity of the enzyme at concentrations up to 55 μM .

L-Leucine was found to be unable to relieve the inhibition of glutamate dehydrogenase by phosphatidylserine (Fig. 2). In contrast the inhibition by cardiolipin was relieved by L-leucine, as shown in Fig. 3. Thus the apparent activation by this amino acid was increased in the presence of cardiolipin (Fig. 3 inset). An L-leucine concentration of 5 mM was found to be sufficient to relieve the cardiolipin inhibition up to 75% (Fig. 4). Thus the observed activation by this amino acid in the

Fig. 4. Relationship between L-leucine activation and cardiolipin inhibition of ox brain glutamate dehydrogenase

The initial velocity of the reaction was determined in the absence and presence of 5 mM-L-leucine and in the presence of various concentrations of cardiolipin, in the range 0–82 $\mu\text{g/ml}$. v_0 represents the velocities in the absence of L-leucine and cardiolipin, v represents the velocities determined in the presence of cardiolipin alone, and v_a represents the velocities in the presence of cardiolipin plus 5 mM-leucine.

presence of cardiolipin is comparable with that observed in the mitochondrial preparations (see Table 2).

Glutamate dehydrogenase from rat has been reported to differ from the more extensively studied ox enzyme (King & Frieden, 1970). However, the effects of cardiolipin and L-leucine on the purified enzyme from rat brain were found to be similar to those observed with the ox brain preparation (results not shown).

The analysis of samples of the purified ox liver and brain glutamate dehydrogenase preparations for phospholipid phosphate revealed none to be detectable. The sensitivity of the assay was such that amounts less than 5 nmol of phospholipid phosphate/mg of protein would not be detected. The presence of this upper-limit amount of phospholipid in the enzyme preparations would result in a maximum addition of 0.5 nmol-phospholipid P to the assay mixtures, since the maximum amount of the pure enzyme present in kinetic studies was 0.25 μg . Determination of the phosphate concentrations of the phospholipid preparations gave values of 1.1 nmol of P/ μg for phosphatidylserine and 0.56 nmol of P/ μg for cardiolipin. Thus the concentration ranges used in the studies shown in Figs. 2–4 were 40–80 μM - and 1.5–46 μM -phospholipid P for phosphatidylserine and cardiolipin respectively, values greatly in excess of the upper limit for the possible presence of phospholipids in the enzyme preparation.

DISCUSSION

Glutamate dehydrogenase activity appears to be strongly inhibited in intact mitochondria (see Greville,

1969; Dodd, 1973). The inhibitory effects of phospholipid binding may contribute to such inhibition. Cardiolipin is a major component of the mitochondrial inner membrane (see Nemat-Gorgani & Dodd, 1977a), and Godinot (1973) has shown the presence of glutamate to increase the association of glutamate dehydrogenase with cardiolipin and to hinder the release of the enzyme from mitochondrial-inner-membrane matrix preparations. In contrast with these findings, Pour-Rahimi & Nemat-Gorgani (1987) reported that glutamate dehydrogenase activity was not significantly inhibited in mitoplast preparations, and that the activity of the enzyme bound to such preparations was increased by L-leucine to a lesser extent than for the unbound enzyme. These results also conflict with the large differences between the sensitivities of the enzyme in intact and disrupted mitochondria and those of purified preparations to activation by L-leucine (McGivan *et al.*, 1973; the present work). The reasons for these apparent discrepancies require further investigation. It is possible that the glutamate dehydrogenase-mitoplastic-membrane preparation or the assay used in the work of Pour-Rahimi & Nemat-Gorgani (1987) may differ too much from the intact system. Thus, for example, the presence of digitonin in that preparation might affect the enzyme-phospholipid interactions.

The inhibition by phosphatidylserine and cardiolipin, but not by phosphatidylcholine, indicates that these effects are not simply a non-specific action of phospholipids. Dodd (1973) also reported phosphatidylcholine to have no significant effects on the activity of the enzyme, but Julliard & Gautheron (1972) reported it to be inhibitory at concentrations between 10 and 110 μM . The ability of L-leucine to relieve the inhibitory effects of cardiolipin, but not those of phosphatidylserine, further indicates that specific, rather than non-specific, interactions are involved.

The differences in the sensitivities of purified preparations of glutamate dehydrogenase and those in disrupted mitochondria to activation by L-leucine are in agreement with the results of McGivan *et al.* (1973), who reported the enzyme to be more markedly activated by that amino acid in intact or disrupted mitochondrial preparations. The loss of sensitivity to L-leucine during the rapid purification procedure used in the present work would be consistent with the removal of a factor modulating the response to that amino acid. Phospholipid analysis of purified preparations of the enzyme revealed that any such material present would result in concentrations present in the enzyme assays that were at least 2000 times lower than the concentrations of cardiolipin found necessary to give 50% inhibition of glutamate dehydrogenase activity.

We have shown preparations of the enzyme which have suffered limited proteolysis during the purification process to differ from the unmodified enzyme in their responses to L-leucine in the presence of inhibitory concentrations of NADH (Couée & Tipton, 1989). However, these effects would not appear to be great enough to account for the large differences in sensitivities observed by McGivan *et al.* (1973), and cannot account

for the effects observed in the present work, where preparations of the enzyme which had not suffered proteolytic modification were compared with disrupted mitochondrial preparations.

The interactions of L-leucine with other allosteric effectors of glutamate dehydrogenase such as NADH and GTP (see Couée & Tipton, 1989) would be expected to contribute to the complex effects of this amino acid in intact systems. However, it appears that the inhibition of the enzyme by cardiolipin, which has also been reported by others (Julliard & Gautheron, 1972; Dodd, 1973; Godinot, 1973; Nemat-Gorgani & Dodd, 1977a,b; Pour-Rahimi & Nemat-Gorgani, 1987) and the specific relief of that inhibition by L-leucine, reported in the present work, may be sufficient to account for the difference between the sensitivities of the isolated enzyme and that in mitochondrial preparations.

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