LIPOIC ACID INHIBITION OF MITOCHONDRIAL MALATE DEHYDROGENASE

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SUMMARY: Porcine heart mitochondrial malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) has been shown to be inhibited by extremely low concentrations of lipoic acid. The actual inhibitor was found to be a high molecular weight substance, which can be separated by gel permeation from the non-inhibitory monomeric form of lipoic acid. This inhibitor has been identified as a polymeric form of lipoic acid.

INTRODUCTION: Lipoic acid (1,2-dithiolane-3-valeric acid), recognized as a cofactor in several α-keto acid dehydrogenase systems, has been previously shown to affect developmental processes in regenerating hydra and planaria, (1) and to affect enzymatic activity in several dehydrogenase enzymes (2). This report concerns a closer inspection of the inhibitory effect of lipoic acid on the enzyme porcine heart mitochondrial malate dehydrogenase (MDH). Lipoic acid solutions are inhibitory at micromolar concentrations; however, removal of a high molecular weight species from the solution by gel permeation significantly reduces the inhibitory effect. On the other hand, the high molecular weight species obtained in this separation exhibits an extremely inhibitory effect on malate dehydrogenase. This species is most likely a polymeric form of lipoic acid, linked through disulfide bonds and identified by its spectral characteristics as reported by Thomas and Reed (3).

This suggests that the observed inhibition of this dehydrogenase enzyme is due to the polymeric form of lipoic acid and not to the monomeric form of the molecule. In addition, this inhibition appears to be competitive with the reduced coenzyme for the enzymatic reaction, NADH.

MATERIALS AND METHODS: Mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig heart as previously described by Gregory, et. al.

(4). Lipoic acid (DL-thioctic acid) was obtained from Sigma.

The enzymatic activity of MDH was determined by following the loss of NADH during the reduction of oxalacetate, monitoring the change in absorbance at 340 nm on a Unicam SP1800 recording spectrophotometer. Initial assay conditions contained 100 mM sodium phosphate buffer, pH 7.5, 0.127 mM oxalacetate, and NADH at 0.16, 0.08, 0.04, or 0.02 mM. To these were added varying concentrations of inhibitor. Activity was determined by adding 1 µl of enzyme (7.1×10^{-6}M) and monitoring the initial change in 340 nm absorbance.

Lipoic acid solutions were prepared by first dissolving the weighed crystals (40 mg) in 1 N NaOH (0.5 ml) by means of a vortex mixer. This procedure was necessary to produce the sodium salt of lipoic acid which is soluble in aqueous solutions. The solution was then diluted to 10.0 ml with 100 mM sodium phosphate buffer, pH 7.5.

Chromatography of the lipoic acid solutions was performed on a Sephadex G-50 column (1.6 x 70 cm) in water. A 2.0 ml aliquot of the lipoic acid solution (2.0×10^{-2}M) was applied to the column and eluted fractions were monitored for absorbance at 250 nm and 330 nm. Fractions exhibiting the maximum absorbance at each of these wavelengths were used for studies of inhibitory effects on MDH enzymatic activity.

To determine monomeric lipoic acid concentration in 100 mM sodium phosphate buffer, a sample of a fraction derived from the portion of the eluant containing only monomeric lipoic acid from the Sephadex G-50 was lyophilized to dryness. The solid was redissolved in 95% ethanol and the concentration of lipoic acid was determined using ε_{330}=150 in 95% EtOH (3). As there was no direct means of determining the concentrations of the polymeric species, fractions eluting from the Sephadex G-50 in the void volume were diluted to the same extent as were the monomeric fractions for studying their effect on enzymatic activity.

RESULTS AND DISCUSSION: The unchromatographed lipoic acid solution at low concentrations (10^{-5} to 2×10^{-6}M) inhibits mitochondrial MDH as shown in

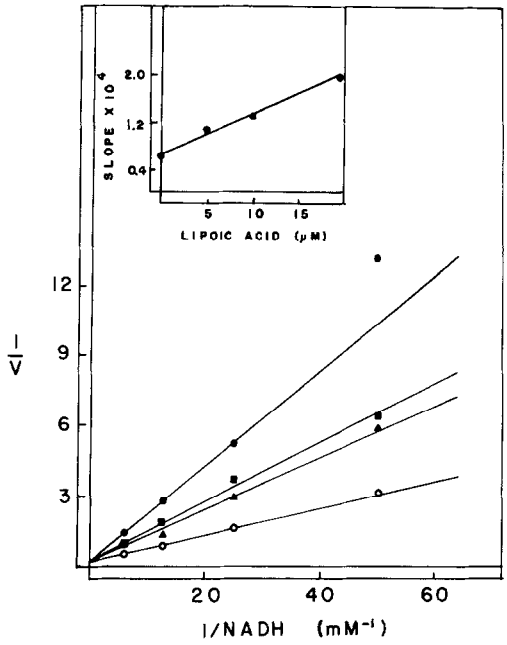

Figure 1

Lineweaver-Burke plot of lipoic acid inhibition of mitochondrial malate dehydrogenase. Initial velocities were determined upon addition of 1 µl of enzyme (7 x 10^{-6}M) to the assay mixture (described in Materials and Methods) containing 4.8 µM (▲), 9.7 µM (■), 19.3 µM (●) or no (○) lipoic acid.

Fig. 1. The inhibition appears to be competitive with the reduced cofactor, NADH, with a calculated K_I value of approximately 5 x 10^{-6}M, assuming all lipoic acid is in the monomeric form.

As can be seen in Fig. 2, there are two species in the original lipoic acid solution which are well separated by chromatography on the Sephadex G-50 column. The ultraviolet spectrum of the peak fraction of early eluting species is characteristic of polymeric lipoic acid (3) with relatively weak and broad absorbance near 250 nm and only low end absorbance in the 330 nm region. The peak fraction of the second eluting species shows characteristic absorption of monomeric lipoic acid with an absorption maximum at 330 nm.

Fig. 3 is the Lineweaver-Burke plot of data showing the effect on MDH activity of the 2 types of materials which were eluted from the Sephadex G-50 column. Both forms of lipoic acid show some inhibitory effect on MDH, but the

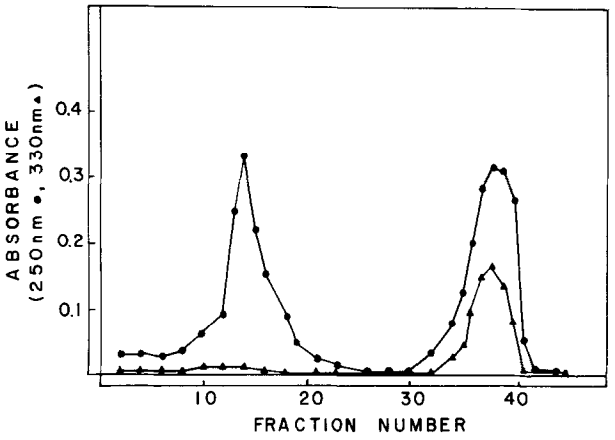

Figure 2

Chromatography of lipoic acid on Sephadex G-50 column. A 2.0 ml aliquoit of lipoic acid (1.9×10^{-2}M) was applied to the column (1.6 x 70 cm) and 3.5 ml fractions were collected. Fractions were monitored for both A_{250} (●) and A_{330} (▲).

effect of high molecular weight species is definitely greater. The curvature of the line showing the effect of isolated polymer on MDH activity is suggestive of some form of cooperativity, but this aspect will need to be more closely investigated before a firm interpretation is suggested.

The inhibitory species in this first peak eluting from the Sephadex G-50 has been identified as polymer from its absorption characteristics, previously described by Thomas and Reed (3). The minimum molecular weight of this form may be in the range of 10,000 to 30,000 since it elutes in the void volume of the Sephadex G-50. It is also probable that this eluting material is a random mixture of different molecular weight polymers, all or only a few of which may be inhibitory.

The value for K_I for the polymeric form of lipoic acid cannot be calculated since the concentration of this species cannot be determined directly; however, this K_I must be less than the 10^{-6}M concentration of monomeric forms which combine to create this polymer. The K_I for solutions containing primarily monomeric species was determined to be 10^{-4}M or greater.

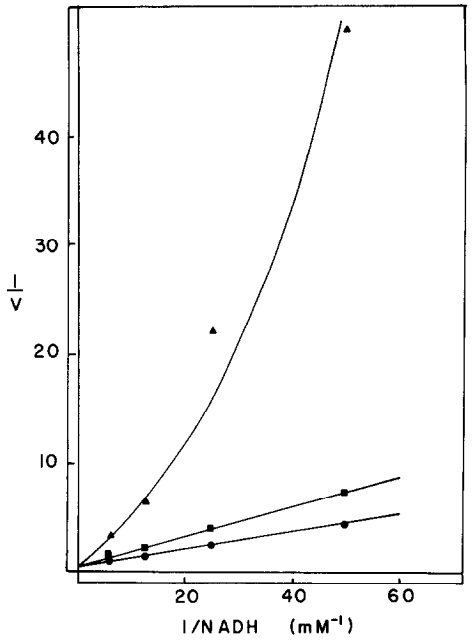

Figure 3

Lineweaver-Burke plot of inhibition of mitochondrial malate dehydrogenase by two lipoic acid species eluting from Sephadex G-50 column. The monomer fraction (A_{330}=0.340) was diluted 1/50 for the assay mixtures; the polymer fraction (A_{330}=0.045) was also diluted 1/50. The effect on initial velocities is shown for monomer (■), polymer (▲) and no lipoic acid (●).

Over a 3-7 day period, only a 10% change (increase) was seen in the absorbance at 330 nm of the unchromatographed solutions. This suggests that under the conditions employed (pH 7.5) some of the polymer may depolymerize to monomer, however apparently not to a large extent. The inhibitory effects of these solutions was affected very little, if any, by the change.

Solutions prepared with lipoic acid which was first dissolved in 1N NaOH, followed by dilution in 100 mM sodium phosphate buffer, pH 7.5, produce larger amounts of the polymer material and were determined to be more inhibitory than those prepared by first dissolving the lipoic acid in 0.1N or 0.5 N NaOH. Gel permeation chromatography of the latter solutions (in 0.1 N or 0.5 N NaOH) demonstrated that little polymeric material was present, and the calculated K_I values for these apparently monomeric solutions were 100 fold

higher than values for those solutions prepared in 1.0 N NaOH. This finding is consistent with the base catalyzed formation of disulfide interchange and hence formation of polymer.

Previous studies of lipoic acid inhibition of several dehydrogenases demonstrated that the K_I for lipoic acid was lowest for MDH (2). The results presented here indicate the inhibitory species is actually a high molecular weight polymeric form of lipoic acid, identified by its absorbance characteristics, which would be present at even lower concentrations than indicated by calculated K_I values. It might be expected that this polymer may have also been responsible for the inhibition of other dehydrogenases in the previous work (2).

Lipoic acid has recently been tested to some extent as a possible antidote to mushroom poisoning from Amanita phalloides (5). Those studies have indicated that when lipoic acid is administered with sufficient glucose to maintain blood sugar levels, the hypoglycemia produced by amanitin toxin (and by lipoic acid alone) is not observed (6). The mechanism of this therapeutic action of lipoic acid in treatment of mushroom poisoning is not yet known, but again, this may be due to either a monomeric or polymeric form. The hypoglycemia observed in administration of lipoic acid may be related to the previously reported lipoic acid inhibition of glucose-6-phosphate dehydrogenase (2). Thus the mechanism of inhibition of other dehydrogenases, especially glucose-6-phosphate dehydrogenase, may give some insight into this antidote activity and the hypoglycemic effect caused by lipoic acid.

In addition, lipoic acid has been shown to inhibit regeneration of tissue in both hydra and planaria (1). A further study is in progress in our laboratory to determine if this observed biological effect is also caused by the polymeric form rather than monomeric lipoic acid. The mechanism of the inhibition of malate dehydrogenase, and perhaps of the developmental process, by such a large polymeric molecule, might be expected to differ from that of small enzyme inhibitors so extensively studies in protein chemistry. Thus an extensive investigation of the mode of interaction of polymer and enzyme will be of prime importance.

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