Stereospecific Reduction of *R*(+)-Thioctic Acid by Porcine Heart Lipoamide Dehydrogenase/Diaphorase

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R(+)-thioctic acid is the naturally occurring cofactor in α-ketoacid dehydrogenases. We show both photometrically by NADH + H^+ oxidation and by HPLC product analysis that this enantiomer is rapidly reduced by NADH + H^+ catalyzed by porcine heart lipoamide dehydrogenase/diaphorase. The racemate exhibits approximately 40% activity as compared to the *R*(+) form while the *S*(−) enantiomer photometrically shows little activity and yields no detectable reduced lipoic acid.

Introduction

Thioctic acid (oxidized lipoic acid; 1,2-dithiolan-3-pentane carboxylic acid) acts as cofactor of α-ketoacid (pyruvate, ketoglutarate) dehydrogenases and transfers two electrons from thiamine-pyrophosphate complex *via* FAD of the dehydrogenase to NAD^+ simultaneously catalyzing the transfer of an acyl moiety from the thiamine complex to the coenzyme A. The molecule exists in two chirally different forms, *R*(+) and *S*(−). The naturally occurring molecule is the *R*(+) enantiomer (Mislow and Meluch, 1956), as suggested by studies on its biosynthesis (Parry and Trainor, 1978; White, 1980a, b). In all biological and biochemical studies performed so far, the commercially available racemic mixture has been used and it has not yet been shown, to our knowledge, that the naturally occurring *R*(+) enantiomer is the preferred substrate for its converting enzyme(s), the lipoamide dehydrogenases/diaphorases.

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Materials and Methods

Chemicals and Biochemicals

Lipoamide dehydrogenase (E.C. 1.8.1.4) from porcine heart (suspension in 3.2 M $(NH_4)_2SO_4$; pH approx. 6; 10.4 mg protein (Biuret)) and NADH (disodium salt) were purchased from Sigma, Munich. EDTA (Titriplex III), acetonitrile (HPLC grade) and the phosphate buffer salts were obtained from Merck, Darmstadt. The enantiomers of thioctic acid (lipoic acid) were a gift from ASTA-Medica AG, Frankfurt.

Methods

The unit activity of lipoamide dehydrogenase was calculated for *R,S*-lipoic acid as substrate (5.6 u/mg at pH 5.9; unit definition: one unit will oxidize 1.0 μmol of β-NADH per min at 25 °C, at the indicated pH and with the appropriate substrate). All photometric assays had a total volume of 2 ml, containing 0.1 M phosphate buffer, pH 5.9, 1 mM EDTA, 200 μM NADH, 500 μM lipoic acid and 0.025 units of lipoic acid dehydrogenase. The *R,S*-racemate of lipoic acid as well as the enantiomers (*R*- and *S*-form of lipoic acid) were tested as substrates in this system. The kinetics were observed at a wavelength of 339 nm during the first 10 min after addition of the enzyme. To control

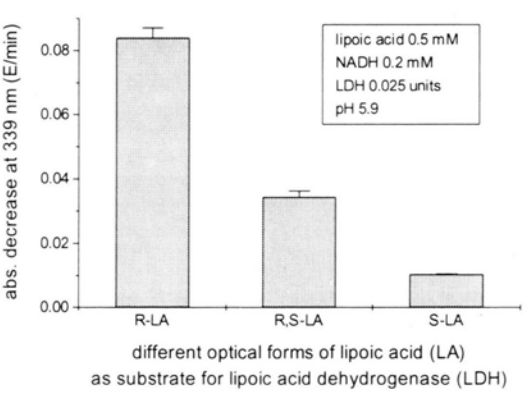

Fig. 1. Oxidation of NADH by lipoic dehydrogenase with different optical forms of lipoic acid as substrate. The measured activity of LDH (based on the molar extinction coefficient ε = 6200 for NADH) was for *R*-LA as substrate 0.0135 ± 0.0005 units, for the racemate *R,S*-LA 0.0055 ± 0.0003 units and for *S*-LA as substrate 0.0016 ± 0.00002 units.

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(verify) the reduction of lipoic acid, 20 µl aliquots of the incubation samples were analyzed with HPLC and the amount of reduced lipoic acid was calculated from a standard curve. HPLC conditions: Beckman HPLC with two 114 M pumps, a 20 µl sample loop, a Waters column heater and a Beckman 160 absorbance detector with a 254 nm filter; range 0.005 was used. Separation of lipoic acid from its reduced form was carried out with a Macherey-Nagel column Nucleosil® 7 C 18 (250/8/4), temperated at 35 °C. The flow rate of the mobile phase, a mixture of a sterile filtered 50 mM phosphate buffer, pH 2.3 (65 V%) with acetonitrile (HPLC grade) (35 V%), was 1 ml per min. Typical retention times were for lipoic acid 10.5 min and for reduced lipoic acid 13.5 min.

Results and Discussion

Oxidation of $NADH+H^+$ by the thioctic acid enantiomers was followed photometrically. The decreases of extinction after 10 min of reaction with the different electron acceptors are shown in Table I and Fig. 1. To verify that a real electron transport has taken place and not an "unspecific" autoxidation, the products have been quantified by HPLC analysis. As shown in Table II, in the presence of 200 µM $NADH+H^+$ approximately 155 µM dihydrolipoate are found after 10 min of reaction corresponding to a 75% utilization of the electron donor. The racemic mixture yielded only *ca.* 60 µM product, corresponding to approximately 40% activity of the *R*(+) form.

Table I. Decrease of the absorption per min at the wavelength 339 nm (see Fig. 1).

R-Lipoic acid	−0.08391 ± 0.00316 E
R,S-Lipoic acid	−0.03439 ± 0.00182 E
S-Lipoic acid	−0.01034 ± 0.00012 E

Mean of $n = 5 \pm$ s.d.

Table II. Reduced lipoic acid after 10 min reaction time.

R-Lipoic acid	155 ± 12 µM
R,S-Lipoic acid	59 ± 8 µM
S-Lipoic acid	out of range

Mean of $n = 3 \pm$ s.d.

As compared to the *R*(+) enantiomer, the *S*(−) form yields no detectable reduced product. The observation that the *S*(−) form exhibits some activity in the photometric test may be due to a certain "wobbling (uncertainty of performance) of the stereospecificity" of the enzyme or to minor impurities of the used thioctic acid samples.

Thioctic acid is widely used as a therapeutic drug against various diseases such as diabetic polyneuropathy and reperfusion injuries. These pharmacological activities are probably due to the antioxidative property of the reduced form in certain cellular compartments (Borbe and Ulrich, 1989). The notion that the racemic mixture is only by about 40% as active as the *R*(+) enantiomer may suggest to clinically test the pure *R*(+) compound instead of the racemic mixture.

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