Biochemical Pharmacology, Vol. 51, pp. 233–238, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02124-8

Reduction of Lipoic Acid by Lipoamide Dehydrogenase

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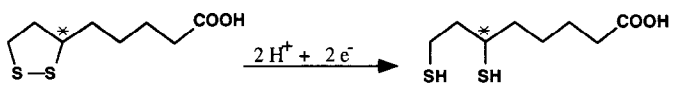
ABSTRACT. Racemic lipoic acid is therapeutically applied in pathologies in which free radicals are involved. The *in vivo* reduction of lipoic acid may play an essential role in its antioxidant effect. It was found that mitochondrial lipoamide dehydrogenase (LipDH, EC 1.8.1.4.) reduces the R-enantiomer 28 times faster than the S-enantiomer of lipoic acid. Moreover, it was observed that the metabolites of lipoic acid, bisnor-, tetranor-, and beta-lipoic acid are poor substrates of LipDH. S-lipoic acid inhibits the reduction of the R enantiomer only at relatively high concentrations. The reduction of R-lipoic acid by mitochondria-rich tissues may proceed smoothly, even if the racemic mixture is applied. This is of importance in elucidating the molecular mechanism of the pharmacotherapeutic effect of lipoic acid. BIOCHEM PHARMACOL 51;3:233–238, 1996.

KEY WORDS. lipoic acid; dihydrolipoic acid; thioctic acid; lipoamide dehydrogenase; antioxidant; enzymatic reduction

$lipS_2$† is the trivial name for 1,2-dithiolane-3-pentanoic acid. Upon reduction, the disulfide-containing ring is opened and 6,8-dimercapto-octanoic acid (DHLA) is formed:



lipoic acid ($LipS_2$) dihydrolipoic acid (DHLA)

This oxidation-reduction process is part of the reaction of several mitochondrial multienzyme complexes in which lipoic acid functions as a cofactor. These complexes are the pyruvate dehydrogenase complex, the α-ketoglutarate dehydrogenase complex, the branched chain oxo acid dehydrogenase complex and the glycine cleavage system [1]. Generally, in these complexes, lipoic acid is involved in energy production and NADH formation. Therapeutically, lipoic acid is used in a variety of diseases including liver cirrhosis, heavy metal intoxication, and diabetic polyneuropathy. Diabetic polyneuropathy particularly is accompanied by free radical-mediated processes caused by high glucose levels. In 1988, it was proposed that the antioxidant activity of lipoic acid might play an important role in its therapeutic efficacy [2]. This hypothesis aroused extensive research into the antioxidant profile of lipoic acid.

DHLA and lipoic acid have been investigated for their scavenging capability of molecules initiating free radical damage [3–6]. Determination of the rate constants for some scavenging reactions appeared to cause experimental problems. For example, in examining hydroxyl radical scavenging the reduction of Fe^{3+} to Fe^{2+} by DHLA accelerates ·OH formation [4]. Also, the indirect antioxidant activity of lipoic acid appears to be important. DHLA can regenerate oxidized vitamin C [5] and oxidized glutathione [7]. DHLA can also interact with chromanoxyl radicals [5, 7]. In several *in vivo* animal models, DHLA seems the protective agent. Neuroprotection was seen by i.p. injection of DHLA 30 min prior to middle cerebral artery occlusion [8]. Lipoic acid itself showed the same size of infarcted area as the control. In animal models for diabetes DHLA, especially and not lipoic acid, improved the parameters depicting the severity of the diabetic state [9, 10]. In most cases, DHLA appears to be at least equipotent or superior to lipoate. Therapeutically, lipoic acid is given in the oxidized racemic form. Therefore, the possible reduction of lipoic acid *in vivo* is of major importance.

The question arises as to which enzyme may be responsible for lipoic acid reduction. A good candidate is the enzyme LipDH (EC 1.8.1.4.). This enzyme is part of the mitochondrial multienzyme complex in which lipoic acid acts as a cofactor. In the complex, the physiologically occurring reaction is the oxidation of a dihydrolipoic acid attached to the E2 component. However, separated from the complex, the reduction of $lipS_2$ has been shown *in vitro* for rat liver [11] and pig heart [12]. The reduction of free, exogenous lipoate by mitochon-

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† *Abbreviations:* DHLA, dihydrolipoic acid; DTNB, 5,5-dithiobis (2-nitrobenzoic acid); GSSG, oxidized glutathione; LipDH, lipoamide dehydrogenase (EC 1.8.1.4); $lipS_2$, lipoic acid; TNB, 5-thio,2-nitrobenzoic acid.

Received 17 May 1995; accepted 4 September 1995.

drial LipDH may occur in two ways. First, it is suggested that in the absence of NAD^+ the attached DHLA, produced during the acylation reaction in the complex, reduces the LipDH. This reduced LipDH may be capable of reducing free lipoate [13]. Second, it may also be possible that the LipDH present in the multienzyme complex at the same time acts as a free lipoate reductase [14]. LipDH may be responsible for the observed reduction of exogenous lipoic acid by mitochondria [15]. Interestingly, in some species LipDH is found outside the enzyme complex, associated with the plasma membrane [16]. Little is known of this LipDH. Probably dithiol-disulfide interchanges may play a general role in membrane-related processes such as the transport of ribose, galactose, and maltose and insulin-stimulated glucose transport.

In this study, the reduction of lipoic acid by mitochondrial LipDH was examined. For the pure enzyme we investigated (i) the stereospecificity, (ii) the substratespecificity, and (iii) the possible competitive inhibition between the enantiomers. The study of these properties is important for determining which enzyme may reduce lipoic acid *in vivo*.

MATERIALS AND METHODS

The kinetic mechanism of the reduction of $lipS_2$ by pig heart LipDH follows the ping pong bi bi mechanism [12].

$$NADH + LipDH_{ox} \rightleftarrows NADH - LipDH_{ox} \quad (1)$$

$$NADH - LipDH_{ox} \rightleftarrows NAD^+ + LipDH_{red} \quad (2)$$

$$LipDH_{red} + lipS_2 \rightleftarrows LipDH_{red} - lipS_2 \quad (3)$$

$$LipDH_{red} - lipS_2 \rightleftarrows LipDH_{ox} + DHLA \quad (4)$$

The reduction of $lipS_2$ and its derivatives was studied on a Pharmacia LKB Ultrospec Plus spectrophotometer. By measuring the decrease in NADH concentration at 380 nm (ϵNADH = 1114.0, ϵlipoic acid = 41.4 M^{-1} cm^{-1}) the initial rate of LipDH reduction (reaction 1 and 2) was determined in the first 60 sec. The initial rate of DHLA formation (reaction 4) was determined in presence of 1.5 mM of the thiol-reagent DTNB [17]. At this concentration the reaction:

$$DHLA + DTNB \rightarrow lipS_2 + 2\ TNB \quad (5)$$

was not rate limiting (data not shown). Moreover DTNB did not react with LipDH under these conditions. TNB formation was followed at 500 nm (ϵTNB = 1100 M^{-1} cm^{-1}) where DTNB absorption was zero. All assays were performed under semi-physiological conditions: a 50 mM KH_2PO_4/KOH buffer (pH = 7.4) containing 110 mM NaCl, in a final volume of 1 mL and kept at 37° with a thermostatically controlled cell chamber of the spectrophotometer. All solutions were stored on ice and equilibrated at 37° for 5 min in a shaking waterbath. The reaction was started by addition of 5 µg LipDH (unless otherwise noted). All experiments were done in triplicate consisting of internal duplicates.

The apparent K_m was computed roughly by analyzing the Lineweaver-Burk plot and fitting of the Michaelis-Menten equation using the Kaleidagraph computer program (Apple Macintosh).

NADH:lipoamide oxidoreductase from pig heart (grade I) and Na_2-NADH (grade II) were obtained from Boehringer, Mannheim, Germany. The specific activity of the different batches of LipDH was determined. Comparisons were only made using experiments with the same batch. DTNB was obtained from Janssen Chimica, Geel, Belgium. The racemates of bisnorlipoic acid, tetranorlipoic acid, dihydrolipoic acid, betalipoic acid, and the enantiomers R-$lipS_2$ and S-$lipS_2$ were kind gifts from ASTA Pharma, Frankfurt, Germany.

RESULTS

Figure 1 shows that the initial rate of the reduction of R-$lipS_2$ depends on the NADH concentration, as would be expected for a ping pong bi bi mechanism. LipDH reduction reached its maximal velocity at NADH concentrations above 0.1 mM. We used 1 mM NADH to further study the reduction of lipoic acid.

The initial rates were measured in presence of DTNB. This had two advantages. First, the $lipS_2$ concentration remained constant (reaction 5) during the measurement. It was, therefore, possible to follow the linear formation of TNB over a longer time period. Second, the possibility of product inhibition by DHLA was prevented. It was observed that 4 mM DHLA decreased the reduction of R-$lipS_2$ (8 mM) by 20% (data not shown) in 2 min.

The reaction rates of lipoic acid and its derivatives were studied at 1.0 mM NADH or by using 1.0 mM NADH plus 1.5

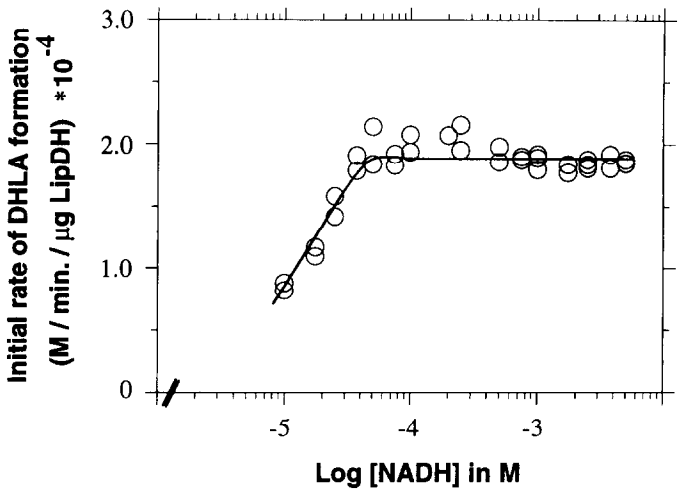

FIG. 1. The dependence of the initial reduction rate on NADH concentration. R-lipoic acid concentration is 20 mM. DHLA formation was monitored within the first 30 sec at 500 nm using 1.5 mM of the thiol-reagent DTNB. The reaction was started by the addition of LipDH. The amount of LipDH varied from 5 µg to 0.1 µg with decreasing NADH concentration. One circle represents the mean of an independent experiment done in triplicate.

mM DTNB. For R-lipS_2, identical initial rates were found when the reaction was followed either by NADH consumption or TNB formation (Fig. 2A). For S-lipS_2, however, the oxidation of NADH was much faster than S-lipS_2 reduction (Fig. 2B). Using the DTNB method, the reduction rate of S-lipS_2 could be determined and compared to the R-lipS_2 reduction (Fig. 3).

Surprisingly, bisnorlipoic acid, tetranorlipoic acid, and beta-lipoic acid (metabolites of lipoic acid [18, 19]) reacted with 1.5 mM DTNB. Therefore, the DTNB method could not be used. By monitoring the decrease in NADH it appeared that, at a 20 mM concentration of the metabolite, the oxidation of NADH was still much higher than the reduction of S-lipS_2 (Table 1).

The influence of the S enantiomer on R-lipS_2 reduction is shown in Fig. 4. Up to a 20-fold excess of S-lipS_2, the reduction rate of lipoic acid was hardly influenced; only a small decrease was found. At higher S-lipS_2/R-lipS_2 ratios DHLA formation became increasingly determined only by the reduction of the S-isomer (Fig. 4A). The reduction of R-lipS_2 decreased with a higher concentration of S-lipS_2 as depicted in Fig. 4B. Therapeutically, the racemate form of lipoic acid was applied. Therefore, the concentration-dependent reduction of the racemate was compared with pure R-lipS_2 (Fig. 5). When the initial reaction rate of racemic lipoic acid was multiplied by a factor 2, it became obvious that the racemic compound showed exactly half the activity of R-lipS_2. No relevant inhibition by the S-isomer on the conversion of the R-isomer was observed here.

DISCUSSION

LipDH activity depends on both its substrates, NADH and lipoic acid. At a concentration above 0.1 mM NADH, the rate of reduction was solely determined by the lipS_2 concentration. From Fig. 1, the K_m for NADH was roughly estimated at 10^{-5} M. This is in the same order of magnitude as the K_m seen in rat liver mitochondria ($6.2 * 10^{-5}$ M; pH = 8, T = 37°) [11].

An example of the dependence of the initial rate of reduction on the concentration of lipoic acid can be seen in Fig. 2. Even at 20 mM, saturation conditions were not reached.

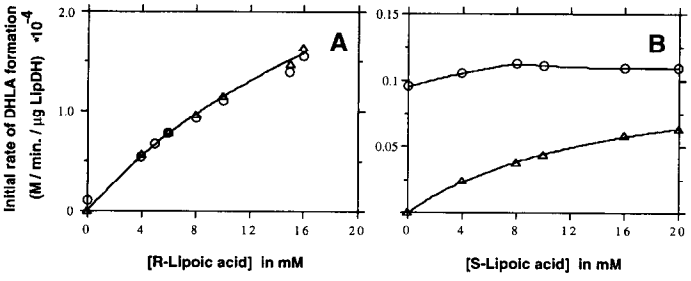

FIG. 2. The dependence of the initial rate of DHLA formation on the concentration of R-lipoic acid (A) and S-lipoic acid (B) at 1.0 mM NADH. The reaction was started by the addition of 5 µg LipDH. Within the first 60 sec, the linear decrease in NADH concentration (○) was monitored at 380 nm and DHLA formation (△) was monitored at 500 nm using the thiol-reagent DTNB.

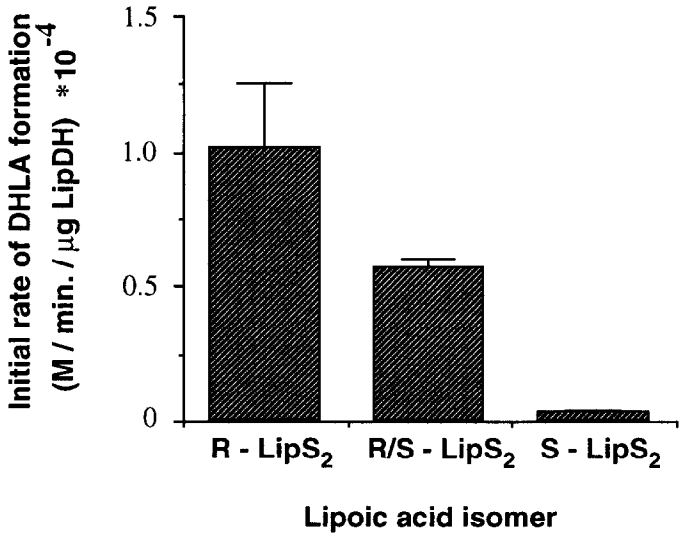

FIG. 3. The stereospecificity of lipoic acid reduction by LipDH. The initial reaction rates were determined using 0.8 mM lipoic acid and 1.0 mM NADH. The reaction was started by the addition of 5 µg LipDH. Within the first 60 sec, DHLA formation was monitored at 500 nm using the thiol-reagent DTNB.

Higher concentrations of lipoic acid could not be used due to the influence of its viscosity and its poor solubility. In addition to reduction of lipoic acid, LipDH also possesses NADH oxidase activity [20]:

$$NADH + O_2 + 2\ H^+ \rightarrow NAD^+ + H_2O_2 \qquad (6)$$

It was found that the oxidase activity interfered with the reduction of S-lipS_2 monitored by NADH consumption (Fig. 2B). We estimate the apparent K_m for R-lipS_2 and S-lipS_2 to be approximately 20 mM. This is approximately 10 times higher than Massey [21] estimated for racemic lipS_2 ($K_m \approx 2$ mM) at pH = 5.9 and $T = 25°$. This difference may be due to the different reaction conditions. Comparing the apparent K_m values for both enantiomers, we found that both apparent K_ms are within the same range, and the reaction rates differ 28-fold. Schempp *et al.* observed an 8-fold difference in reaction rate at [NADH] = 0.2 mM, pH = 5.9, and $T = 25°$ [22] and Suzuki *et al.* reported a 15-fold difference at [NADH] = 0.1 mM, pH =

TABLE 1. Initial rate of NADH oxidation by LipDH in presence of different lipoic acid analogs

Lipoic acid analog	NADH consumption (M/min/µg LipDH) * 10^{-4}	SEM * 10^{-4}
No substrate	0.083	0.009
Tetranorlipoic acid	0.086	0.005
Bisnorlipoic acid	0.097	0.010
Beta-lipoic acid	0.321	0.047
S-lipoic acid	0.116	0.009
R-lipoic acid	1.656	0.176

In presence of a lipoic acid analog (20 mM), the oxidation of 1.0 mM NADH by LipDH was monitored at 380 nm. Immediately after the addition of 5 µg LipDH, the linear decrease in absorbance was measured for 30 sec as described in Materials and Methods.

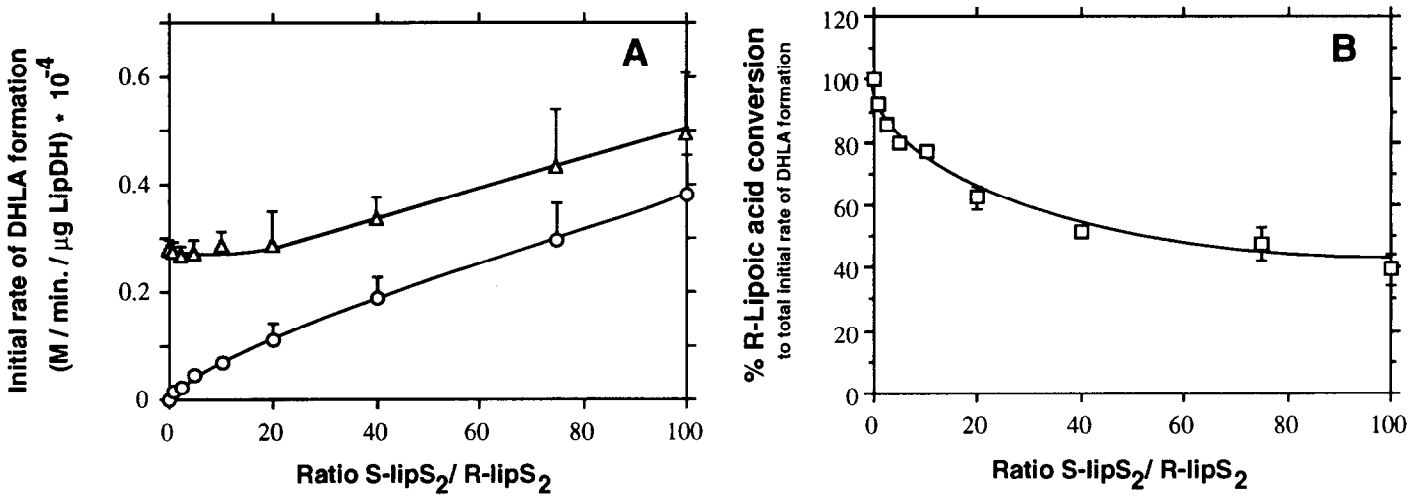

FIG. 4. The inhibition of S-lipoic acid on the reduction of R-lipoic acid by LipDH. (A) The initial rate of DHLA formation by S-lipoic acid with (△) or without (○) 0.2 mM R-lipoic acid. Within the first 60 sec, DHLA formation was monitored at 500 nm using the thiol-reagent DTNB in presence of 1.0 mM NADH. The reaction was started by addition of 5 µg LipDH. (B) The influence of S-lipoic acid on R-lipoic acid reduction, calculated from the results depicted in (A). The percentage inhibition is calculated per experiment. Thereafter the average was determined from 3 separate experiments.

7.4 (*T* unknown) [23]. It is difficult to explain these different observations. The estimated K_ms depend on reaction conditions, such as NADH concentration, pH, and temperature. However, it is supposed that the relative difference in reaction rate between the isomers is not influenced much by the difference in conditions. Probably the purity of S-lipoic acid explains the differences.

As early as 1960, stereospecificity was suggested by Massey [21]. Using different methods and conditions, Suzuki and Packer [23] and Schempp *et al.* [22] demonstrated stereospecificity for the reduction of lipS_2. Yang and Frey [24] and Arscot *et al.* [25] showed stereospecificity for DHLA oxidation. The stereospecificity of LipDH may be useful to investigate enzymatic DHLA formation *in vivo*. Recently, Handelman *et al.* [26] found support for *in vivo* reduction. They demonstrated DHLA formation after addition of lipoic acid to human cells in tissue culture. Lipoic acid reduction is presumably enzymatic. This was supported by the findings of Fuchs and Milbradt [27]. Given in the diet, the naturally occurring R-enantiomer of lipoic acid inhibited glucose oxidase-induced dermatitis, but the S-enantiomer was only marginally protective. Glucose oxidase-induced dermatitis is probably mediated *via* hydrogen peroxide and subsequent free radical formation. The difference between the isomers can be explained by a stereospecific reduction of lipoic acid into the more potent antioxidant DHLA. This observed stereospecificity points not to a pure chemical, but to an enzymatic reduction. The cytosolic enzyme GSSG reductase can also reduce lipoic acid [28]. In this case, however, an opposite stereospecificity is expected; GSSG reductase shows a 2.4-fold greater preference for S-lipS_2 than for R-lipS_2. An enzyme inhibitor of LipDH that discerned LipDH and glutathione reductase would be an interesting pharmacological tool to determine the relative contribution of both enzymes. Unfortunately, no inhibitor of LipDH is known yet.

Remarkably, 1.5 mM DTNB reacts with bisnor-, tetranor-, and beta-lipoic acid. Apparently DTNB is not entirely specific for measuring thiol groups [17]. Measuring NADH consumption, it was found that beta-lipoic acid is a slightly better substrate than S-lipoic acid for LipDH. In addition, it was discovered that bisnor- and tetranorlipoic acid are poor substrates (Table 1). Interestingly, Suzuki and Packer [23] found that beta-lipoic acid showed similar LipDH activity as bisnor- and tetranorlipoic acid. However, they applied different incubation conditions and used a DTNB method. The DTNB

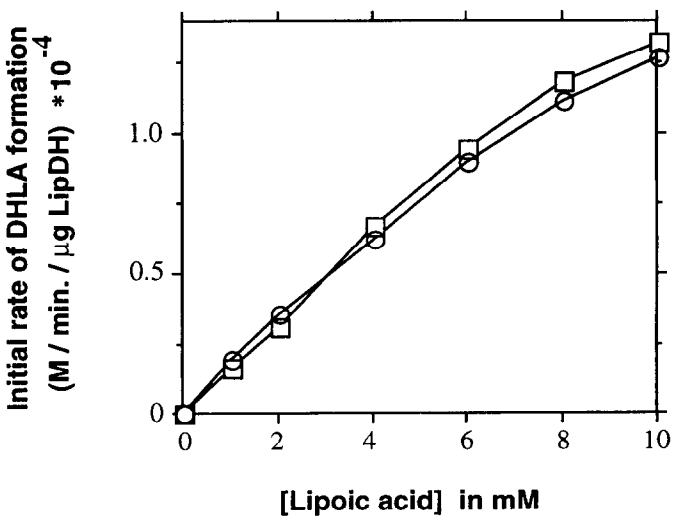

FIG. 5. The initial rate of DHLA formation from racemic lipoic acid (○) and R-lipoic acid (□). For comparison, the observed rate of racemic lipoic acid was multiplied by 2. Within the first 60 sec, DHLA formation was monitored at 500 nm using the thiol-reagent DTNB in presence of 1.0 mM NADH. The reaction was started by addition of 5 µg LipDH. The figure shows a typical example of one experiment in which all conditions are the same.

method is based on the formation of thiols from the substrates. Initially, the reduction of beta-lipoic acid will result in the formation of lipoic acid. Therefore, in the case of beta-lipoic acid no thiol is formed, which explains the underestimated LipDH activity compared to the higher activity determined by NADH consumption. Shortening of the side chain of the lipoyl moiety (lipoic acid attached to lysine) decreases the reaction rate. This is consistent with the observations of Massey in 1960 [21]. Furthermore, the metabolites are poor substrates for the enzyme GSSG reductase. The V_{max} of bisnor- and tetranorlipoic acid are 19% and 7.5%, respectively of the V_{max} of racemic lipoic acid [28]. Assuming that one of these enzymes is responsible for the reduction, it is expected that the reduced metabolites will be present in very low amounts. Therefore, the contribution of these compounds to the antioxidant profile of lipoic acid is expected to be of minor importance [29]. These compounds may only play a role in scavenging HOCl [30].

S-lipS_2 appeared to be a poor substrate of LipDH with an estimated apparent K_m in the same order as for R-lipS_2. Therefore, it is likely that this compound occupies the active site and inhibits the reduction of R-lipS_2. Remarkably, even in a 20-fold excess, no inhibition of the R-lipS_2 reduction is observed (Fig. 4). Under a racemic condition in which the R-lipS_2:S-lipS_2 ratio is 1:1, S-lipS_2 does not influence the reduction of R-lipS_2 (Fig. 5). This indicates that the estimated apparent K_ms do not reflect the affinity constants of S-lipS_2 and R-lipS_2 for LipDH. As mentioned above, lipoic acid is administered as a racemic mixture. The pharmacokinetics of the compounds are different, although the differences are minor (personal communication ASTA, cited with permission). Therefore, it may be postulated that a 20-fold excess of S-lipS_2 over R-lipS_2 *in vivo* is not reached after administration of racemic lipoic acid. Moreover, endogenous R-lipS_2 is present. This indicates that the inhibition of LipDH by the S-enantiomer is of minor physiological relevance. GSSG reductase reduces R-lipS_2 and S-lipS_2 equally effectively. This means that in cytosol, where GSSG-reductase is active, both R-lipS_2 and S-lipS_2 contribute to antioxidant activity. In contrast, LipDH-dependent reduction may result in an antioxidant activity of lipoic acid that originates from only half of the racemic mixture; the S-isomer can be regarded as isomeric ballast.

In conclusion, the reduction of lipoic acid seems of importance for its pharmacotherapeutic effect. To answer the question as to which enzyme reduces lipoic acid *in vivo*, it is necessary to know the properties of enzymes that reduce lipoic acid. We, therefore, studied mitochondrial LipDH. The stereospecificity and the influence of S-lipoic acid differ for LipDH and GSSG-reductase. It is not yet known whether LipDH has the same characteristics in plasma membranes as mitochondrial LipDH. More information is needed on the biological effects of lipoic acid in relation to its stereospecific reduction. This may help to elucidate the enzymatic reduction and, thereby, the mode of action of lipoic acid. To support such studies we described the following properties of mitochondrial LipDH under semi-physiological conditions: (i) LipDH shows stereospecificity for the naturally occurring R enantiomer, (ii) the metabolites bisnor-, tetranor-, and beta-lipoic acid are poor substrates for LipDH, and (iii) S-lipS_2 inhibits the reduction of R-lipS_2 only in relatively high concentrations. The reduction of lipoic acid by mitochondria-rich tissues may proceed smoothly, even if the racemic mixture is applied. This may be an important mechanism of reduction of lipoic acid *in vivo*.

We would like to thank Dr. A. de Kok from the Agricultural University in Wageningen, the Netherlands, for his valuable advice. We acknowledge ASTA Pharma, Frankfurt, Germany, who supplied us with the compounds.

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