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INTERACTION OF α-LIPOIC ACID ENANTIOMERS AND HOMOLOGUES WITH THE ENZYME COMPONENTS OF THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

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Abstract—Lipoic acid (α-lipoic acid, thioctic acid) is applied as a therapeutic agent in various diseases accompanied by polyneuropathia such as diabetes mellitus. The stereoselectivity and specificity of lipoic acid for the pyruvate dehydrogenase complex and its component enzymes from different sources has been studied. The dihydrolipoamide dehydrogenase component from pig heart has a clear preference for *R*-lipoic acid, a substrate which reacts 24 times faster than the *S*-enantiomer. Selectivity is more at the stage of the catalytic reaction than of binding. The Michaelis constants of both enantiomers are comparable (K_m = 3.7 and 5.5 mM for *R*- and *S*-lipoic acid, respectively) and the *S*-enantiomer inhibits the *R*-lipoic acid dependent reaction with an inhibition constant similar to its Michaelis constant. When three lipoic acid homologues were tested, *RS*-1,2-dithiolane-3-caproic acid was one carbon atom longer than lipoic acid, while *RS*-bisnorlipoic acid and *RS*-tetranorlipoic acid were two and four carbon atoms shorter, respectively. All are poor substrates but bind to and inhibit the enzyme with an affinity similar to that of *S*-lipoic acid. No essential differences with respect to its reaction with lipoic acid enantiomers and homologues exist between free and complex-bound dihydrolipoamide dehydrogenase. Dihydrolipoamide dehydrogenase from human renal carcinoma has a higher Michaelis constant for *R*-lipoic acid (K_m = 18 mM) and does not accept the *S*-enantiomer as a substrate. Both enantiomers of lipoic acid are inhibitors of the overall reaction of the bovine pyruvate dehydrogenase complex, but stimulate the respective enzyme complexes from rat as well as from *Escherichia coli*. The *S*-enantiomer is the stronger inhibitor, the *R*-enantiomer the better activator. The two enantiomers have no influence on the partial reaction of the bovine pyruvate dehydrogenase component, but do inhibit this enzyme component from rat kidney. The implications of these results are discussed.

Key words: pyruvate dehydrogenase complex; dihydrolipoamide dehydrogenase; α-lipoic acid; enantiomers; enzyme kinetics; diabetes mellitus

Lipoic acid (α-lipoic acid, thioctic acid, 1,2-dithiolane-3-valeric acid) is an essential cofactor of the 2-oxoacid dehydrogenase multienzyme complexes: the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex, and the branched-chain 2-oxoacid dehydrogenase complex. It is covalently bound to a lysyl-residue of dihydrolipoamide acyltransferase, one of the three enzyme components of these multienzyme complexes. The lipoyl-lysyl-residue is located on a flexible peptide loop, the lipoyl domain, which mediates, like a swinging arm, the interaction between the different catalytic centres of the three enzyme components of these multienzyme complexes: 2-oxoacid dehydrogenase, dihydrolipoamide acyltransferase, and dihydrolipoamide dehydrogenase [1–3]. The lipoyl domain accepts the acyl intermediate from the 2-oxoacid dehydrogenase component and transfers it to coenzyme A. In the course of this reaction lipoic acid is reduced to dihydrolipoic acid and its dithiolane ring opened. Reoxidation and ring closure is catalysed by the dihydrolipoamide dehydrogenase component. The reduction equivalents released by this reaction are taken up by a reactive dithiol group and a FAD cofactor at the active site and finally accepted by NAD^+ [4]. While both the pyruvate and 2-oxoacid dehydrogenase complexes have their specific 2-oxoacid dehydrogenase and dihydrolipoamide acyltransferase components, the dihydrolipoamide dehydrogenases of both multienzyme complexes are identical [5].

Lipoic acid has an asymmetric centre at its C6 position. Previous studies in bacteria have shown that *R*-lipoic acid is a natural enantiomer [6, 7]. This finding raises the question whether non-physiological *S*-lipoic acid is also accepted as a substrate and cofactor of the 2-oxoacid dehydrogenase complexes or whether it acts as an inhibitor or antagonist. In this study we investigated the influence of the enantiomers and of some homologues of lipoic acid on the partial enzymatic reactions of the dihydrolipoamide dehydrogenase and pyruvate

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§ Abbreviations: K_m, Michaelis constant; K_i, inhibition constant; k_{cat}, catalytical constant; V, maximum velocity.

dehydrogenase component and on the overall reaction of the pyruvate dehydrogenase complex from different mammalian sources.

Besides the covalently bound lipoyl cofactor dihydrolipoamide dehydrogenase also accepts free lipoic acid from the cytoplasm as a substrate controlling the equilibrium between its oxidized and reduced states. Free lipoic acid serves as an antioxidant and radical scavenger for cells [8–10]. Consequently, dihydrolipoamide dehydrogenase exists in the cell both as an enzyme component of 2-oxoacid dehydrogenase complexes and in a free, soluble form. Lipoic acid is applied in the therapy of various diseases, especially those accompanied by polyneuropathia such as diabetes mellitus [11, 12]. The molecular basis of its action is not yet clear. Both its role as an essential cofactor of multienzyme complexes controlling key reactions of energy metabolism and its function as antioxidant and radical scavenger must be considered. Dihydrolipoamide dehydrogenase is involved in both processes and the study of the interaction of lipoic acid enantiomers and homologues with this enzyme should contribute to the understanding of the basis of the therapeutic efficiency of lipoic acid.

MATERIALS AND METHODS

Chemicals. *R*- and *S*-lipoic acid, *RS*-bisnorlipoic (1,2-dithiolane-3-propanoic) acid and *RS*-tetranorlipoic (1,2-dithiolane-3-carboxylic) acid were gifts from ASTA Medica AG (Frankfurt, Germany). *R*- and *S*-dihydrolipoic acid were synthesized from their respective lipoic acid enantiomers by reduction with sodium borohydride according to the method of Reed *et al.* [13]. *RS*-1,2-dithiolane-3-caproic acid was synthesized according to Thomas and Reed [14]. The pyruvate dehydrogenase complex from porcine heart was obtained from Sigma (Deisenhofen, Germany). Dihydrolipoamide dehydrogenase (diaphorase, EC 1.8.1.4) from porcine heart and substrates and cofactors for the enzyme tests were bought from Boehringer Mannheim (Germany). Polyethylene glycol 6000, the chemicals for polyacrylamide gel electrophoresis and 2,6-dichlorophenolindophenol were obtained from Serva (Heidelberg, Germany). Buffer substances were from Merck (Darmstadt, Germany).

Enzyme preparations. The pyruvate dehydrogenase complex from bovine heart was purified according to the procedure of Stanley and Perham [15]. The pyruvate dehydrogenase complexes from bovine and rat kidney were partially purified by applying this procedure only until the second polyethylene glycol precipitation step. The pyruvate dehydrogenase complex from human renal carcinoma was also isolated according to this method, but only to the first polyethylene glycol precipitation. The pyruvate dehydrogenase complex from *Escherichia coli* was purified according to Bisswanger [16].

The purity of the enzyme preparation was verified by polyacrylamide gel electrophoresis according to Laemmli and Favre [17], with the protein concentration being determined by the method of Bradford [18].

Enzyme tests. The overall activity of the pyruvate dehydrogenase complex was tested spectrophotometrically by the reduction of NAD at 340 nm and 30° according to Schwartz and Reed [19]. The partial reaction of the pyruvate dehydrogenase component (EC 1.2.4.1) was measured with 2,6-dichlorophenolindophenol as electron acceptor at 30° according to Khailowa *et al.* [20]. Dihydrolipoamide dehydrogenase activity was tested with lipoic acid and NADH at 30° as described in Bergmeyer [21]. The reverse reaction was determined with dihydrolipoic acid and NAD following the procedure of Schmincke-Ott and Bisswanger [22]. Enzyme activities for all enzyme tests were defined as μmol product formed per min. Enzyme kinetic data were plotted in the Hanes–Woolf diagram applying linear regression analysis. In most cases, each measurement was repeated three times. Standard deviations were calculated according to the least squares method [23].

RESULTS

Reaction of lipoic acid enantiomers with free and complex-bound dihydrolipoamide dehydrogenase from porcine heart

The enantiomers of lipoic acid were tested for their ability to act as substrates in the catalytic reaction of dihydrolipoamide dehydrogenase both in its free and complex-bound form. For these experiments the concentrations of both the respective lipoic acid enantiomer and the NADH cosubstrate were varied. The result of such a bisubstrate kinetic analysis is shown for the reaction of *R*-lipoic acid with dihydrolipoamide dehydrogenase from the bovine heart pyruvate dehydrogenase complex in Fig. 1. Plotted in a Hanes–Woolf plot a series of straight lines results intersecting at a common point to the left of the ordinate. Such a pattern is indicative of a sequential mechanism for this enzyme reaction. Free dihydrolipoamide dehydrogenase from porcine heart gave similar results, indicating the absence of influence from the aggregational state of the enzyme. Similar patterns in bisubstrate kinetics were also obtained with the *S*-enantiomer and the racemic mixture of lipoic acid as substrates. Here, however, there were considerable differences in the kinetic constants (Table 1). The Michaelis constant of *S*-lipoic acid is slightly higher than that of the *R*-enantiomer, but the maximum velocities differ remarkably. *R*-lipoic acid has a 24-fold higher maximum velocity than the *S*-form. The enantiomers differ in their catalytic efficiency k_{cat}/K_m by a factor of 36, with *R*-lipoic acid the far better substrate. The maximum velocity of the racemic mixture falls between those of the enantiomers. The K_m values for NADH are essentially the same for all three substrates.

A similar stereoselectivity to that observed for the reduction of lipoic acid by dihydrolipoamide dehydrogenase was also found for the reverse reaction, the oxidation of dihydrolipoic acid by NAD. The Michaelis constants for *R*-dihydrolipoic acid (K_m = 1.8 mM) and *S*-dihydrolipoic acid (K_m = 4.3 mM) differ by a factor of two, with the maximum velocity for the *R*-enantiomer being more than 10-

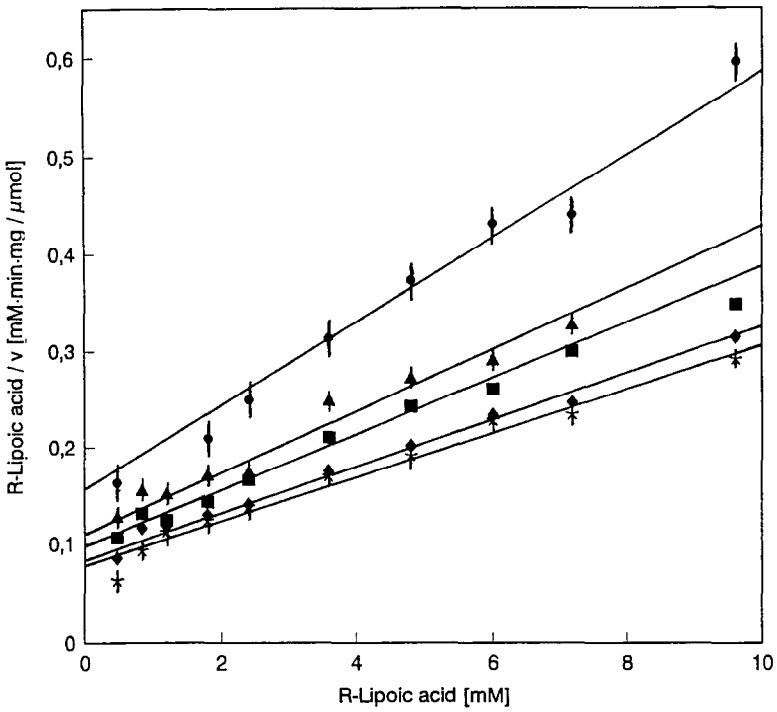

Fig. 1. Hanes–Woolf presentation of the bisubstrate kinetics of dihydrolipoamide dehydrogenase from the bovine heart pyruvate dehydrogenase complex. The enzyme reaction was tested with different amounts of *R*-lipoic acid as indicated at the abscissa scale. The NADH concentrations used were: ●, 5 μM; ▲, 10 μM; ■, 15 μM; ◆, 25 μM; ✳, 50 μM. Purified enzyme preparation (0.4 nkat/mL) was taken for the enzyme test.

Table 1. Kinetic constants of dihydrolipoamide dehydrogenase from porcine heart*

Substrate	$K_{m,\mathrm{Lip}}$ (mM)	$K_{i,\mathrm{Lip}}$ (mM)	$K_{m,\mathrm{NADH}}$ (μM)	V (μmol/min/mg)
R-Lipoic acid	3.7 ± 0.1	—	7.5 ± 0.31	192 ± 2
S-Lipoic acid	5.5 ± 0.35	4.5 ± 0.3	6.0 ± 0.29	7.9 ± 0.1
RS-Lipoic acid	6.5 ± 0.15	—	8.7 ± 0.33	56 ± 1.6
RS-Dithiolanecaproic acid	7.3 ± 0.13	3.2 ± 0.1	†	7.0 ± 0.05
RS-Bisnorlipoic acid	5.7 ± 0.1	10.8 ± 0.5	†	0.9 ± 0.03
RS-Tetranorlipoic acid	10.0 ± 0.2	‡	†	2.9 ± 0.07

* $K_{m,\mathrm{Lip}}$ and $K_{i,\mathrm{Lip}}$ are the Michaelis and inhibition constants, respectively, for the respective lipoic acid enantiomers and homologues; $K_{m,\mathrm{NADH}}$ is the Michaelis constant for NADH, determined in the presence of the respective lipoic acid enantiomer; and V is the maximum velocity determined for saturating amounts of the respective substrate.

† Not determined.

‡ No measurable inhibition.

fold higher than that for the *S*-enantiomer (not shown).

Inhibition of dihydrolipoamide dehydrogenase by S-*lipoic acid*

From their similar Michaelis constants it can be expected that the two enantiomers possess similar affinities to the enzyme. Therefore, if both enantiomers are present at the same time as in the racemic mixture, the less active *S*-lipoic acid should prevent the more active *R*-enantiomer from binding to the catalytic centre, resulting in a reduction of enzymatic activity. The influence of *S*-lipoic acid on the activity of dihydrolipoamide dehydrogenase was studied in terms of dependence on *R*-lipoic acid (Fig. 2A). With increasing amounts of the *S*-enantiomer the data deviate from straight lines in the Hanes–Woolf plot in a way characteristic of the simultaneous action of a strong and a weak substrate [23]. If the turnover of the *S*-enantiomer in the absence of the

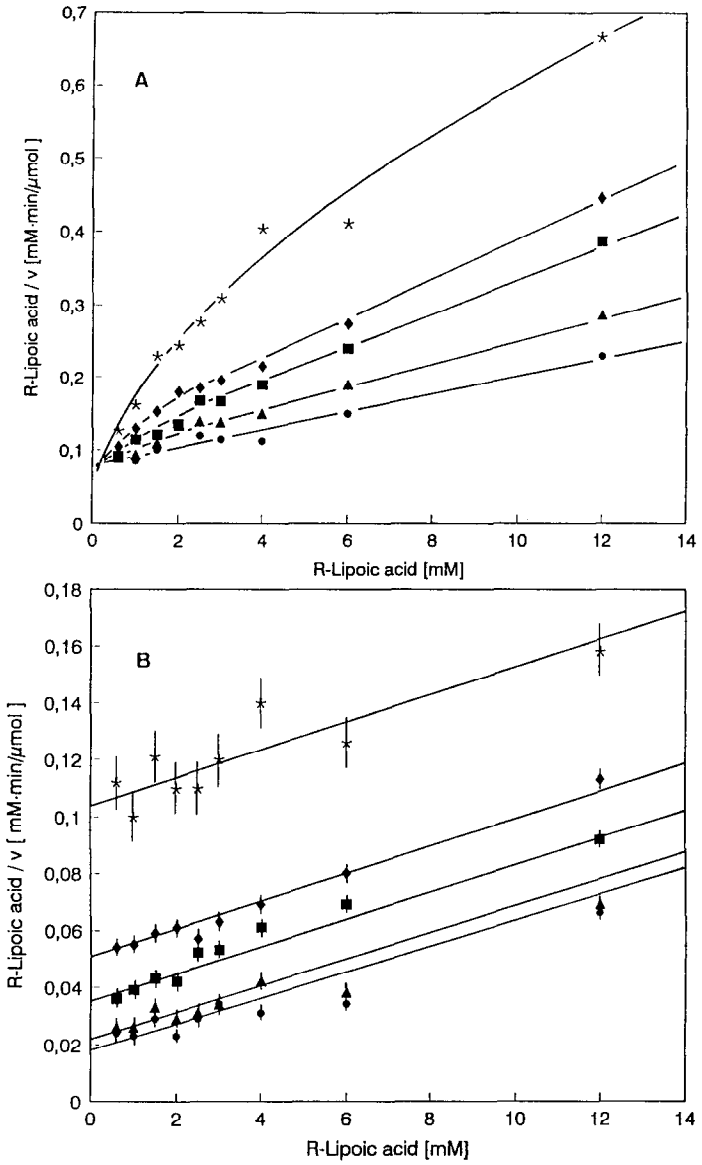

Fig. 2. Influence of *S*-lipoic acid on the enzymatic reaction of dihydrolipoamide dehydrogenase from porcine heart with *R*-lipoic acid. The concentrations of *S*-lipoic acid were: ●, 0 mM; ▲, 1 mM; ■, 3 mM; ◆, 6.2 mM; ✳, 12.4 mM. 0.7 μg of purified dihydrolipoamide dehydrogenase per mL test mixture were applied. The reaction was measured at 35°. A, Hanes–Woolf plot; B, as with A, data corrected for the turnover of the *S*-lipoic acid.

R-enantiomer was subtracted from the data in Fig. 2A, parallel lines were obtained as expected for a competitive inhibition mechanism (Fig. 2B). The inhibition constant for *S*-lipoic acid obtained from these data is in the same range as its Michaelis constant (Table 1).

Reaction of lipoic acid enantiomers with dihydrolipoamide dehydrogenase from human renal carcinoma

The stereospecificity of the dihydrolipoamide dehydrogenase component from partially purified human renal carcinoma pyruvate dehydrogenase complex was tested; only *R*-lipoic acid showed a measurable activity with this enzyme. The Michaelis constant (K_m = 18 mM) was considerably higher than that of the enzyme from porcine heart. The *S*-enantiomer was inactive as a substrate and its inhibitory effect weak, whereas 6 mM *S*-lipoic acid inhibited the enzyme reaction by 30% in the presence of 1.2 mM *R*-lipoic acid as substrate. The inhibition constant is approximately 30 mM (data not shown).

Reaction of lipoic acid homologues with dihydrolipoamide dehydrogenase

The substrate specificity of dihydrolipoamide

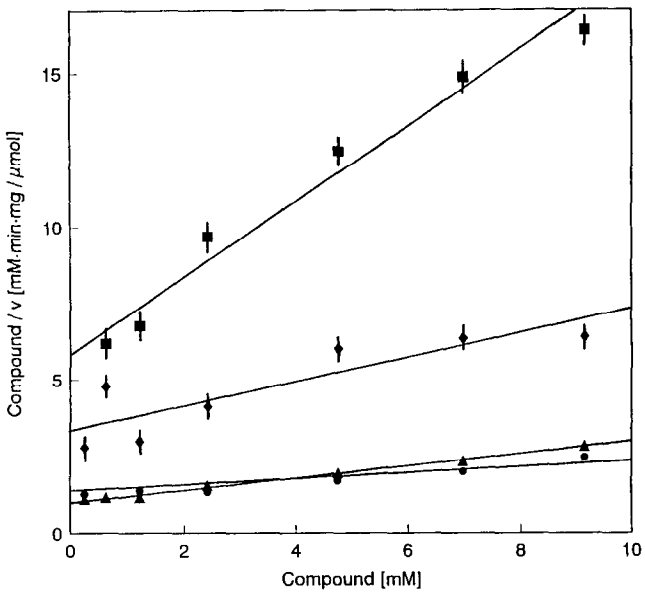

Fig. 3. Hanes–Woolf plot of the dependence of the dihydrolipoamide dehydrogenase reaction on ●, *S*-lipoic acid; ▲, *R*/*S*-1,2-dithiolane-3-caproic acid; ◆, *R*/*S*-bisnorlipoic acid; and ■ *R*/*S*-tetranorlipoic acid. The reaction was measured with 8 μg of purified dihydrolipoamide dehydrogenase from porcine heart.

dehydrogenase was studied with respect to the length of the carbon chain. Bis- and tetranorlipoic acid are degradation products of lipoic acid generated by β-oxidation. Compared to lipoic acid their aliphatic chains are two and four carbon atoms shorter, respectively, while 1,2-dithiolane-3-caproic acid is the homologue one carbon atom longer. The homologues were available only as racemic mixtures. All three compounds were accepted as substrates by dihydrolipoamide dehydrogenase, but they were less active than *R*-lipoic acid (Fig. 3, Table 1). *R*/*S*-1,2-dithiolane-3-caproic acid was comparable to *S*-lipoic acid both with respect to its K_m and *V* values. *R*/*S*-bis- and tetranorlipoic acid showed only a weak turnover, though their Michaelis constants were in the same order of magnitude as that of the other compounds.

With the exception of *R*/*S*-tetranorlipoic acid all homologues inhibit dihydrolipoamide dehydrogenase competitively with respect to *R*-lipoic acid. The inhibition constants are similar to their Michaelis constants (Table 1).

Influence of the lipoic acid enantiomers and homologues on the overall reaction of the bovine pyruvate dehydrogenase complex

When present in the test mixture for the overall reaction of the pyruvate dehydrogenase complex from bovine heart, both lipoic acid enantiomers acted as inhibitors (Fig. 4). The inhibitory effect of the *S*-enantiomer was more pronounced (41% at 1 mM of the *S*-enantiomer) than that of the *R*-form (25% at 1 mM of the *R*-enantiomer). Similar inhibitory effects on the overall reaction were shown by *R*/*S*-1,2-dithiolane-3-caproic acid, *R*/*S*-tetranorlipoic acid and *R*/*S*-bisnorlipoic acid, the latter being the most efficient inhibitor (Fig. 4).

Acetaldehyde is an inhibitor of the overall reaction, acting at the dihydrolipoyl acetyltransferase step [24]. Since the effect of free lipoic acid on the overall reaction is probably due to an interaction with the active site of this enzyme component, the inhibition of lipoic acid enantiomers was studied in the presence of 3.5 μM acetaldehyde. This concentration caused a 25% reduction in the overall reaction. Under these conditions the inhibitory effect of both enantiomers was reduced. Only 10% inhibition with 1 mM *R*-lipoic acid and 18% inhibition with 1 mM *S*-lipoic acid were observed. Acetaldehyde and free lipoic acid clearly compete for the same binding site.

Influence of lipoic acid enantiomers and homologues on the overall reaction of the pyruvate dehydrogenase complex from the rat

Different behaviour has been observed with the pyruvate dehydrogenase complex from rat kidney. Even at low concentrations both lipoic acid enantiomers activated the overall reaction (Fig. 5). *R*-Lipoic acid is the more potent activator: 1 mM of this enantiomer stimulated the reaction up to 167%, while the *S*-enantiomer activated it to 116%. *R*/*S*-bis- and tetranorlipoic acid were still inhibitors, while low amounts of *R*/*S*-1,2-dithiolane-3-caproic acid activated the enzyme reaction and this activation became reversed at higher concentrations (Fig. 5).

Influence of lipoic acid enantiomers and homologues on the overall reaction of the pyruvate dehydrogenase complex from Escherichia coli

The bacterial pyruvate dehydrogenase complex

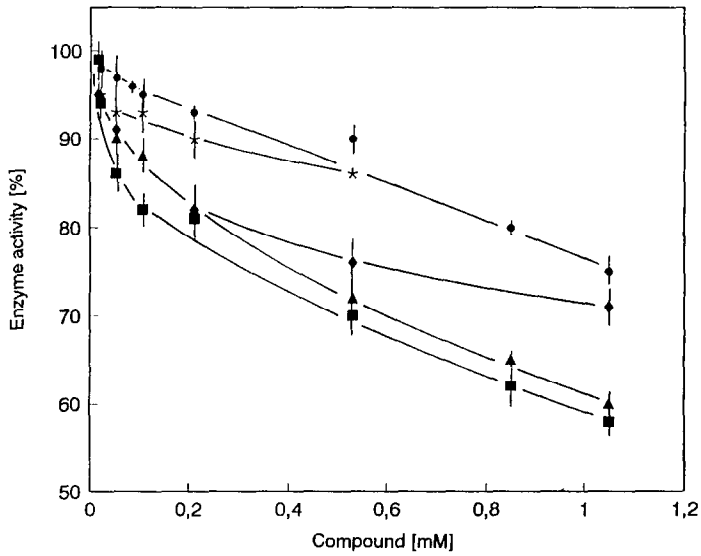

Fig. 4. Influence of lipoic acid enantiomers and homologues on the overall reaction of the pyruvate dehydrogenase complex from bovine heart. ●, *R*-lipoic acid; ▲, *S*-lipoic acid; ■, *R/S*-bisnorlipoic acid; ◆, *R/S*-tetranorlipoic acid; ✳, *R/S*-1, 2-dithiolane-3-caproic acid. The overall reaction was measured with 40 μg of the bovine heart pyruvate dehydrogenase complex, the rate in the absence of effectors (22 nmol/min) being defined as 100%.

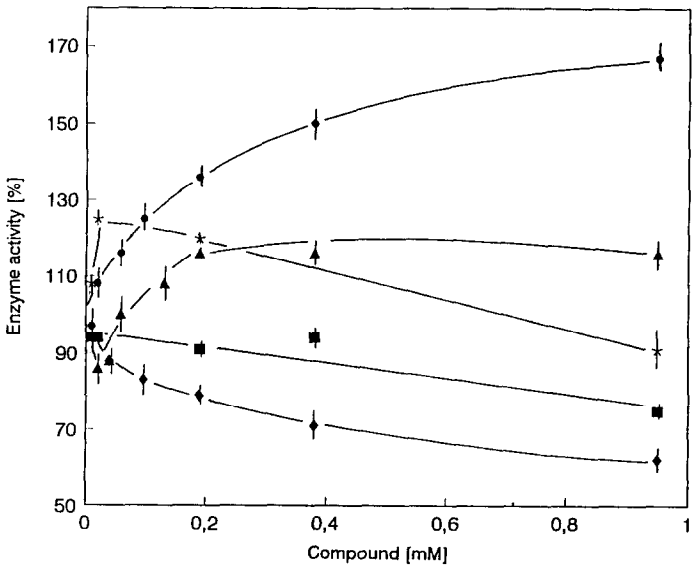

Fig. 5. Influence of lipoic acid enantiomers and homologues on the overall reaction of the rat pyruvate dehydrogenase complex. ●, *R*-lipoic acid; ▲, *S*-lipoic acid; ■, *R/S*-bisnorlipoic acid; ◆, *R/S*-tetranorlipoic acid; ✳, *R/S*-1, 2-dithiolane-3-caproic acid. The overall reaction was measured with 125 μg of the pyruvate dehydrogenase complex from rat kidney. The rate in the absence of effectors (5 nmol/min) has been defined as 100%.

from *E. coli* is comparable to the mammalian enzyme complex with respect to its catalytic mechanism and its structural organization, though its complex structure is simpler and the regulatory enzyme components responsible for controlling overall enzyme activity by reversible phosphorylation are lacking [1]. With this enzyme complex the activation of the overall reaction by the free lipoic acid enantiomers was even more pronounced than with the rat kidney enzyme complex. Figure 6 shows that 0.1 mM *R*-lipoic acid activated the overall reaction to nearly 300%, with the same amount of *S*-lipoic

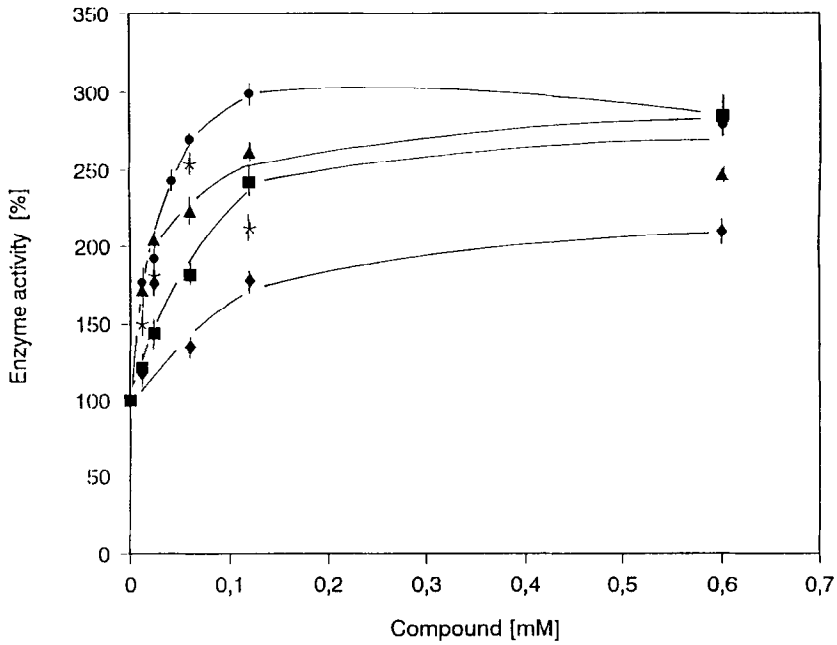

Fig. 6. Influence of lipoic acid enantiomers and homologues on the overall reaction of the pyruvate dehydrogenase complex from *E. coli*. ●, *R*-lipoic acid; ▲, *S*-lipoic acid; ■, *R/S*-bisnorlipoic acid; ◆, *R/S*-tetranorlipoic acid; ✳, *R/S*-1, 2-dithiolane-3-caproic acid. The overall reaction was measured with 35 μg of the purified enzyme complex from *E. coli*. The rate in the absence of effectors (21 nmol/min) has been defined as 100%.

acid activating it to 260%. The homologues of lipoic acid also activated the overall reaction. *R/S*-bisnorlipoic acid was the most efficient: 0.1 mM of this compound stimulated overall activity by a factor of 2.4.

Interaction of lipoic acid enantiomers with the pyruvate dehydrogenase component

The pyruvate dehydrogenase component should carry a specific lipoyl binding site for the transfer of the substrate intermediate from thiamine diphosphate to the lipoyl cofactor. Free lipoic acid may compete with the covalent lipoyl cofactor and may accept both the acetyl intermediate and the electrons being converted to acetyl-dihydrolipoic acid. The partial reaction of the pyruvate dehydrogenase component can be tested separately with pyruvate as substrate in the presence of an artificial electron acceptor such as dichlorophenolindophenol. If the above assumption is true, free lipoic acid should act in this partial reaction as electron acceptor as well. Therefore, this reaction was tested applying the lipoic acid enantiomers as electron acceptors. However, neither the *R*- nor the *S*-enantiomer showed measurable activities in this reaction.

Though a direct transfer of the acetyl intermediate to free lipoic acid could not be demonstrated, specific binding of this compound to the pyruvate dehydrogenase component should at least influence its reaction with dichlorophenolindophenol as electron acceptor. With the enzyme component from the bovine kidney pyruvate dehydrogenase complex no effect could be observed. However, both enantiomers inhibited this reaction when tested with the rat kidney pyruvate dehydrogenase complex (Fig. 7). The *S*-enantiomer was more efficient, showing 45% inhibition at a concentration of 1.2 mM, while the same amount of *R*-lipoic acid showed only 31% inhibition.

DISCUSSION

Previous studies have demonstrated that the *R*-enantiomer of lipoic acid is preferentially accepted by the pyruvate dehydrogenase complex from *E. coli* and it has been concluded that this form represents the physiological enantiomer [6, 7]. The results presented here show that this is also true for the mammalian pyruvate dehydrogenase complex. Although the covalent incorporation of the lipoic acid enantiomer into the central lipoyl cofactor of the enzyme complex could not directly be demonstrated, the clear preference of the dihydrolipoamide dehydrogenase component for the *R*-form supports the assumption that *R*-lipoic acid is the naturally relevant cofactor for mammalians as well.

Because of the clear stereoselectivity for *R*-lipoic acid the potential role of the *S*-enantiomer in racemic mixtures in therapeutical applications must be considered. *S*-lipoic acid may act either as a poor substrate or as an antimetabolite blocking the functions of active *R*-lipoic acid. Our results demonstrate that both possibilities occur in

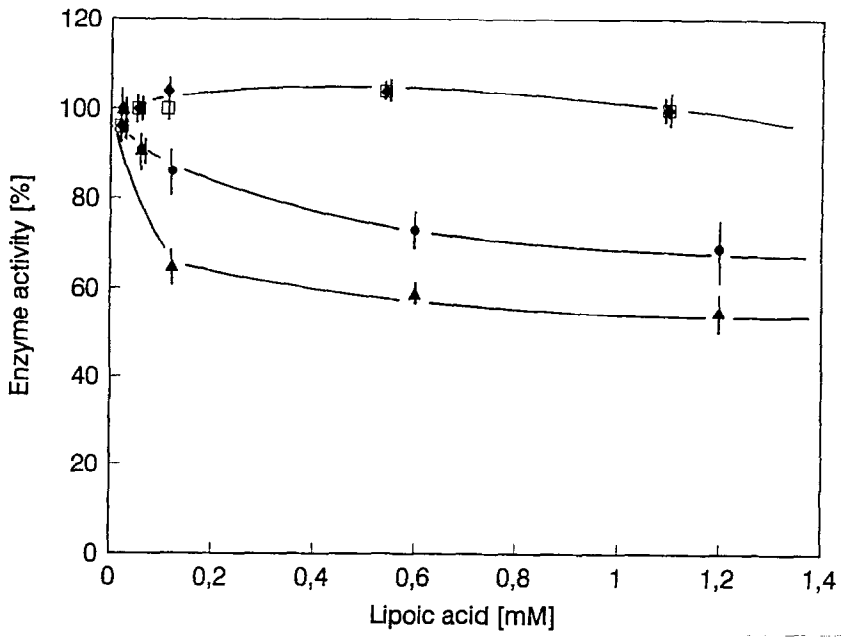

Fig. 7. Influence of lipoic acid enantiomers on the partial reaction of the pyruvate dehydrogenase component from the pyruvate dehydrogenase complex from bovine kidney (□, *R*-lipoic acid; ◆, *S*-lipoic acid), and from rat kidney (●, *R*-lipoic acid; ▲, *S*-lipoic acid). 170 μg of the respective enzyme preparation per mL test mixture were applied. The rate in the absence of effectors (30 nmol for the bovine and 18 nmol for the rat enzyme complex) has been defined as 100%.

parallel. The enantiomers exhibit similar affinities to dihydrolipoamide dehydrogenase, given the comparable Michaelis and inhibition constants of both compounds. They each compete for the substrate binding site at the catalytic centre and the less active *S*-lipoic acid prevents binding of the more active *R*-form and causes a reduction in the reaction rate.

In addition to dihydrolipoamide dehydrogenase's function in the reoxidation of the reduced lipoyl cofactor in the overall reaction of the α-oxoacid dehydrogenase complexes, it plays a role in the control of the redox equilibrium between oxidized and reduced free lipoic acid to protect the cell against damage produced by oxidation and radicals [8–10]. In therapeutic applications of the racemic mixture both enantiomers should be equally efficient in carrying out this function; under normal conditions the slow regeneration of the *S*-enantiomer by dihydrolipoamide dehydrogenase may be sufficient. Under stress conditions however, this slow turnover leads not only to a decrease in the pool of available antioxidants, but also inhibits the fast regeneration of the *R*-form. When taken together, these effects may produce a serious deficiency in the antioxidative potential of the cell.

Similar values for the Michaelis and inhibition constants were obtained for all compounds tested. It is clear that substrate specificity and stereoselectivity for binding to dihydrolipoamide dehydrogenase is relatively poor. Catalysis, on the other hand, appears to be subject to strict structural requirements, since only *R*-lipoic acid is converted at a considerable rate. These kinetic results are confirmed by studies on the structure of the active centre of dihydrolipoamide dehydrogenase on the basis of the resolved X-ray structure [25]. The substrate binding site is formed by a hydrophobic groove. The carboxy group of the substrate is attached to polar residues at one end of this groove. Thus, neither the length of the aliphatic chain nor the configuration at the asymmetric centre is essentially determinative for binding. Crucial, however, are the distance and the relative orientation of the dithiolane ring to the essential dithiol group at the distal end of the groove, which is involved in the transfer of electrons from the substrate to the FAD cofactor and to NAD (Raddatz and Bisswanger, manuscript in preparation).

That *S*-lipoic acid is not a substrate of human dihydrolipoamide dehydrogenase may be due to the remarkably weak affinity of this enzyme for both enantiomers. The ratio between the Michaelis constant of *R*-lipoic acid and the inhibition constant of the *S*-form is the same as for the pig heart enzyme, but the absolute values of these constants in both organisms differ by a factor of five. Thus, the activity of human dihydrolipoamide dehydrogenase with *S*-lipoic acid falls below the sensitivity of the enzyme test.

A ping-pong mechanism has been postulated for dihydrolipoamide dehydrogenase both from bacteria and mammalian sources. Upon oxidation of NADH to NAD, electrons are transferred to the dithiol-FAD centre at the active site of the enzyme. After dissociation of NAD the reduced enzyme

intermediate remains. In a second step the electrons are accepted by lipoic acid under reduction to dihydrolipoic acid [22, 26]. Our kinetic analysis, however, favours a sequential mechanism; one which requires the simultaneous binding of both NADH and lipoic acid to the active site, assuming direct interaction between the substrates as a prerequisite for the catalytic process. Such a mechanism has already been suggested by Veeger *et al.* [27] for the bacterial enzyme. Though it cannot be excluded that dihydrolipoamide dehydrogenases from bacteria and mammals obey different mechanisms, elements of both types may contribute to the actual mechanism of dihydrolipoamide dehydrogenase in such a way that the reduced enzyme intermediate can also exist without bound substrates although their simultaneous presence may favour electron transfer via the dithiol-FAD centre.

The inhibition of the overall reaction of the pyruvate dehydrogenase complex from bovine heart by *RS*-lipoic acid has already been demonstrated [28]. It has been shown that this effect occurs at the dihydrolipoamide acetyltransferase component, possibly at the binding site of the lipoyl cofactor at the active centre. This assumption is supported by the observation that acetaldehyde, which also attacks the dihydrolipoamide acetyltransferase component [24], reduces the degree of inhibition by lipoic acid. More difficult to understand is the stimulating effect of lipoic acid on the rat and the *E. coli* pyruvate dehydrogenase complexes. A similar effect has been observed by Köplin *et al.* [29] for the enzyme complex from rat brain. Two possibilities are to be considered: a distinct activation site for lipoic acid presumably at the dihydrolipoamide acetyltransferase component or a direct participation of free lipoic acid in the overall reaction sequence of the pyruvate dehydrogenase complex comparable to that of the covalent lipoyl cofactor. Free lipoic acid is accepted as a substrate by both dihydrolipoamide acetyltransferase and the dihydrolipoamide dehydrogenase component of the enzyme complex. If lipoic acid is also able to participate in the partial reaction of the pyruvate dehydrogenase component the overall reaction should also proceed with lipoic acid as a dissociable cofactor. However, it could not be demonstrated that lipoic acid acts as an acceptor for electrons and acetyl residues in the pyruvate dehydrogenase reaction. Only the inhibition of this reaction by lipoic acid was observed, but even this effect requires a specific interaction of lipoic acid with the pyruvate dehydrogenase component. It is remarkable that this inhibition could only be observed with the rat pyruvate dehydrogenase complex, whose overall reaction is stimulated by free lipoic acid, but not with the insensitive bovine enzyme complex. Since the test for the partial reaction of the pyruvate dehydrogenase component is relatively insensitive, a small turnover of lipoic acid will be not detectable, but may become visible in the more sensitive overall reaction.

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