Using lipoate enantiomers and thioredoxin to study the mechanism of the 2-oxoacid-dependent dihydrolipoate production by the 2-oxoacid dehydrogenase complexes

V. Bunik[a,*], A. Shoubnikova[a], S. Loeffelhardt[b], H. Bisswanger[b], H.O. Borbe[c], H. Follmann[d]

[a]*A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation*
[b]*Physiological Chemistry Institute, Tuebingen University, Tuebingen, Germany*
[c]*ASTA Medica AG, Frankfurt, Germany*
[d]*Department of Biochemistry, Kassel University, Kassel, Germany*

Received 19 June 1995

Abstract The thioredoxin-catalyzed insulin reduction by dihydrolipoate was applied to study the 2-oxoacid:lipoate oxidoreductase activity of 2-oxoacid dehydrogenase complexes. The enzymatic and non-enzymatic mechanisms of the transfer of reducing equivalents from the complexes to free lipoic acid (α-lipoic acid, 6,8-thiooctic acid) were distinguished using the high stereoselectivity of the complex enzymes to the *R*-enantiomer of lipoate. Unlike these enzymes, thioredoxin from *E. coli* exibited no stereoselectivity upon reduction with chemically obtained dihydrolipoate. However, coupled to the dihydrolipoate production by the dehydrogenase complexes, the process was essentially sensitive both to the enantiomer used and the dihydrolipoyl dehydrogenase activity of the complexes. These results indicated the involvement of the third complex component, dihydrolipoyl dehydrogenase, in the 2-oxoacid-dependent dihydrolipoate formation. The implication of the investigated reaction for a connection between thioredoxin and the 2-oxoacid dehydrogenase complexes in the mitochondrial metabolism are discussed.

Key words: 2-Oxoacid dehydrogenase complex; Thioredoxin; Insulin; 2-Oxoglutarate; Pyruvate; Lipoate enantiomer; Lipoate analog

Introduction

Recently we have started the investigation of the interplay between dehydrogenases of 2-oxoacids and thioredoxins [1]. The dehydrogenases are multienzyme mitochondrial complexes known to utilize pyruvate, 2-oxoglutarate or branched chain 2-oxoacids in the sequence of Reactions 1–3 [2]:

$$\text{2-oxoacid + (S-S)-lip-E2 ---- E1} \rightarrow CO_2 + \text{(acyl-S-SH)-lip-E2} \quad (1)$$

$$\text{(acyl-S-SH)-lip-E2 + CoA} \leftarrow \text{E2} \rightarrow \text{acylCoA + } (SH)_2\text{-lip-E2} \quad (2)$$

$$(SH)_2\text{-lip-E2} + NAD^+ \leftarrow \text{E3} \rightarrow \text{(S-S)-lip-E2 + NADH + H} \quad (3)$$

where E1 is a thiamindiphosphate-dependent dehydrogenase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; and lip represents the lipoyl residue functioning in the complexes covalently bound to the E2 component.

Thioredoxins belong to a family of small redox proteins involved in a variety of physiologically important functions due to their ability to serve as hydrogen donor or to reduce disulfides in proteins [3].

Our study was induced by the discovery of the specific mitochondrial thioredoxin [4] and the known fact that the similar thioredoxin of *E. coli* can be effectively reduced by dihydrolipoate [5]. Because the latter is an intermediate of the 2-oxoacid oxidation occuring in mitochondria, we checked whether the organelle-specific redox transformation of thioredoxin may be coupled to the 2-oxoacid oxidation. Indeed, the *E. coli* thioredoxin becomes reduced at the expense of 2-oxoacid oxidized by mitochondrial 2-oxoacid dehydrogenase complexes [1]. This occurs through dihydrolipoate formed if NAD^+, the terminal electron acceptor in the complex-catalyzed reaction, is omitted and replaced by free lipoate. In this case Reaction 3 of the overall 2-oxoacid oxidation is substituted by Reaction 4:

$$(SH)_2\text{-lip-E2} + \text{(S-S)-lip} \leftrightarrow \text{(S-S)-lip-E2} + (SH)_2\text{-lip} \quad (4)$$

where the covalently bound lipoyl cofactor becomes reoxidized by free lipoic acid.

Coupling 2-oxoacid oxidation to the thioredoxin redox cycle with insulin [1] provides an assay for characterizing the lipoate-reductase activity of the 2-oxoacid dehydrogenase complexes. This system was used in the present work to reveal the mechanism of Reaction 4.

Reaction 4 may be catalyzed by E3, since this component is able to store the reducing equivalents by the FAD-Cys-Cys-center at its active site [6]. Thus, reduced lipoyl cofactor and free lipoate may bind, one after the other, to the same site of E3, exchanging their reducing equivalents. Alternatively, uncatalyzed thiol-disulfide exchange between the E2-bound dihydrolipoate and free lipoate may occur.

The mechanisms were distinguished using the high stereoselectivity of E3 to the *R*-enantiomer of lipoate [7–9]. We showed, that unlike this enzyme, the *E. coli* thioredoxin exhibits no stereoselectivity upon reduction with chemically obtained stereomers of dihydrolipoate. The coupled system, however, was essentially sensitive both to the enantiomer used and the E3 activity of the complexes, pointing to the involvement of E3 in the dihydrolipoate formation in Reaction 4.

Our present results indicate that the native organization of

*Corresponding author.

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; CoA, Coenzyme A.

SSDI 0014-5793(95)00904-3

the 2-oxoacid dehydrogenase complexes, including all three catalytic components, is necessary for the reduction of lipoic acid, while non-enzymatic thiol-disulfide exchange between the complex-bound and free lipoic acid is unable to support the observed reaction.

2. Materials and methods

The 2-oxoglutarate and pyruvate dehydrogenase complexes were obtained from pig heart according to [10] applying the modifications described in [1].

2-Oxoacid-dependent reduction of thioredoxin was determined in a Cary-219 spectrophotometer by the insulin turbidimetric assay [5] as described in [1]. The reaction was measured in 0.1 M potassium phosphate pH 7.0 containing 0.7 mg/ml insulin and 0.15 mg/ml thioredoxin against the blank with all the components but thioredoxin. In addition to this the test mixture for the 2-oxoglutarate dehydrogenase-dependent reaction contained 0.049 U of the 2-oxoglutarate dehydrogenase complex activity (measured with NAD^+ in the standart assay mixture), 1.5 mM 2-oxoglutarate, 0.6 mM CoA and 3 mM lipoate or analogs. Correspondingly, the pyruvate dehydrogenase-dependent reaction mixture contained 0.010 U of the pyruvate dehydrogenase complex activity (measured with NAD^+), 15 mM pyruvate, 0.3 mM CoA, 0.76 mM magnesium chloride, 0.15 mM thiamin diphosphate and 3 mM lipoate or analogs.

R- and *S*-Dihydrolipoate were freshly prepared from their oxidized forms by sodium borohydride reduction by the following procedure. 0.5 ml of 2.5% sodium borohydride in water was added to 30 mg of the corresponding lipoic acid enantiomer dissolved in 0.5 ml of 0.1 M Tris-HCl buffer pH 8.5. After 30 min incubation at room temperature 0.25 ml of 1 M potassium phosphate containing 0.2 N HCl was added. The mixture was left to stand at room temperature for 15 min and 1 ml of acetone was added. The completion of the reaction and the concentration of dihydrolipoate formed were determined by DTNB titration. For measuring the thioredoxin-mediated insulin reduction this dihydrolipoate solution was diluted 20-fold.

Inhibition of 2-oxoglutarate dehydrogenase complex by diphenyliodonium chlorid was studied after 80 min incubation of the enzyme with the inhibitor in the presence of 0.2 mM NADH, followed by gel-filtration on the PD-10 column with Sephadex G-25 medium (Pharmacia) for removing the excess of diphenyliodonium chlorid and NADH.

The 2-oxoglutarate and pyruvate dehydrohenase complex activities were determined in 0.1 M potassium phosphate at pH 7.0 and 7.6, respectively, with 2 mM 2-oxoglutarate or pyruvate, 0.1 mM CoA, 1 mM NAD^+, 0.1 mM thiamin diphosphate and 1 mM magnesium chloride. The E1 activity of 2-oxoglutarate dehydrogenase was assayed with 0.7 mM ferricyanide and 1 mM 2-oxoglutarate in 0.1 M potassium phosphate pH 6.3. The E3 activity was determined with 0.2 mM NADH and 1 mM *R,S*-lipoate in 0.1 M potassium phosphate pH 6.3.

Thioredoxin of *E. coli* was from Calbiochem; insulin, *R,S*-lipoic acid, thiamin diphosphat, CoA were from Serva; 2-oxoglutarate, pyruvate and DTNB - from Merck, diphenyliodonium chloride was from ICN. The lipoate enantiomers and analogs were from ASTA Medica AG, Frankfurt.

3. Results

Reactions of the pyruvate and 2-oxoglutarate dehydrogenase complexes (Reactions 1–2) coupled to the thioredoxin-insulin assay in the presence of lipoate enantiomers and analogs are shown in Fig. 1. In this test system dihydrolipoate accumulated according to Reaction 4 reduces thioredoxin (Reaction 5):

$$\text{(SH)2-lip} + \text{(S-S)-thioredoxin} \leftrightarrow \text{(SH)2-thioredoxin} + \text{(S-S)-lip} \quad \textbf{(5)}$$

Reduced thioredoxin catalyzes reduction of insulin in Reaction 6:

$$\text{(SH)2-thioredoxin} + \text{(S-S)-Insulin} \rightarrow \text{(SH)2-Insulin} + \text{(S-S)-thioredoxin} \quad \textbf{(6)}$$

Reduced insulin precipitates in the reaction medium and the increase in the optical density is followed (Fig. 1). The reaction proceeds at a considerable rate both with the pyruvate (Fig. 1a) and 2-oxoglutarate (Fig. 1b) dehydrogenase complexes only in the presence of the *R*-enantiomer of lipoate (curves 1), while no reaction could be observed with the *S*-enantiomer (curves 2).

To differentiate whether this outcome reflected only the stereospecificity of the lipoate reduction (Reaction 4) or whether the subsequent thioredoxin reduction (Reaction 5) also contributed to the observed stereoselectivity, the thioredoxin-mediated insulin precipitation (Reactions 5 and 6) was studied in the presence of chemically obtained *R*- or *S*-dihydrolipoate. The results of this experiment are shown in Fig. 2. Thioredoxin had no preference for the *R*-enantiomer in insulin reduction (curve 1), rather *S*-dihydrolipoate appeared tdo be slightly more efficient (curve 2). Obviously, the stereoselectivity of the 2-oxoacid-dependent insulin precipitation (Fig. 1) is not determined by any thioredoxin stereospecificity. Thus, it is the step of lipoate reduction (Reaction 4) which is stereoselective.

Structural homologs bis- and tetranorlipoate, with carbon chains being 2 and 4 atoms, respectively, shorter than lipoate, were also tested with the 2-oxoacid dehydrogenase complexes in the thioredoxin-mediated insulin precipitation assay (Fig. 1a,b, curves 3–4). These analogs, like *S*-lipoate, showed only

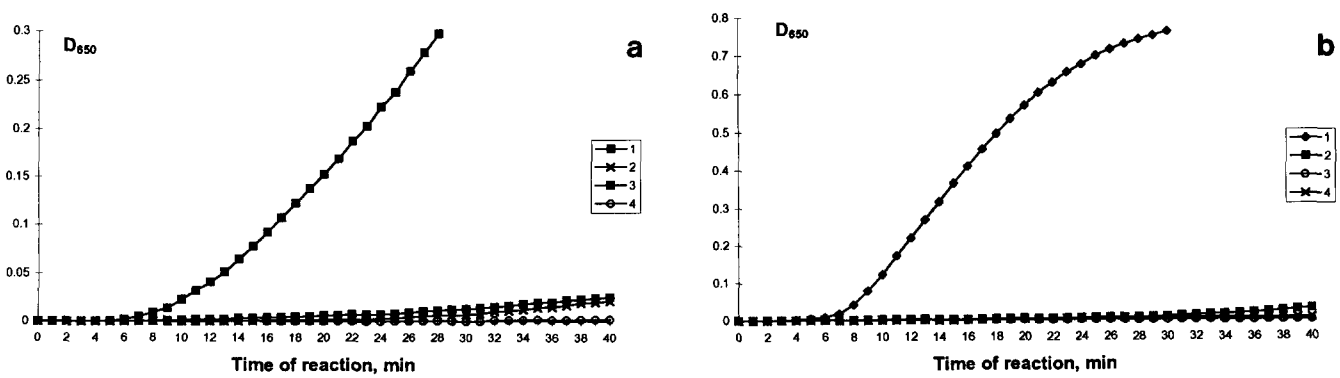

Fig. 1. Insulin reduction by *E. coli* thioredoxin coupled to the pyruvate (a) or 2-oxoglutarate (b) dehydrogenase complexes in the presence of lipoate and analogs. 1, *R*-lipoate; 2, *S*-lipoate; 3, bisnorlipoate; 4, tetranorlipoate. See section 2 for experimental conditions.

3–5% of the E3 activity with the *R*-enantiomer. Their weak activities as substrates in the dihydrolipoate dehydrogenase reaction correlate with their inefficiency in the 2-oxoacid-dependent thioredoxin reduction: the insulin precipitation in the presence of analogs is negligible in comparison to that with *R*-lipoate (Fig. 1).

The dependence of the reduction of free lipoate in the insulin reduction test on the E3 activity of the 2-oxoacid dehydrogenase complexes was further supported by experiments in which E3 was specifically inhibited by diphenyliodonium chlorid. This compound is an inhibitor of FAD-dependent dehydrogenases, forming a tight complex with FAD upon its reduction during a catalytic process [11–13]. Indeed, treatment of the 2-oxoglutarate dehydrogenase complex with the inhibitor alone produced no inhibition. However, incubation with diphenyliodonium chlorid in the presence of NADH led to the concomitant loss of the E3 and overall activities of the complex, while the E1 activity was not decreased (Table 1). The insulin reduction test demonstrated that the activity of the diphenyliodonium chloride-treated 2-oxoglutarate dehydrogenase complex in dihydrolipoate production was lost to the same extent as its E3 activity: compared to the native enzyme complex, the inhibited one showed the five times slower rate of the insulin precipitation, correspondent to the five-fold inhibition of the E3 activity (Table 1).

4. Discussion

The results presented in this work ruled out the possibility of the effective non-enzymatic thiol-disulfide exchange between the E2-bound and free dihydrolipoate, since there is no difference in the redox potentials of both lipoic acid enantiomers and such a reaction should not depend on the nature of the enantiomer. Moreover, all used compounds with dithiolane ring are to undergo redox transformation with similar efficiency, but considerable reduction proceeds only with *R*-lipoic acid, pointing to the enzymatic catalysis of the process (Fig. 1).

Dihydrolipoyl dehydrogenase is known to be highly specific to *R*-lipoate, showing practically no activity with the *S*-enantiomer [7–9]. Taking into account the normal course of the catalytic steps in the 2-oxoacid dehydrogenase complexes, with E2-bound dihydrolipoate reducing E3, our data point to the fact that the E3 component of these enzyme complexes must catalyze Reaction 4. According to this mechanism, the E2-bound dihydrolipoate reduces E3, resulting in the formation of the E2-bound lipoate and the two-electron reduced E3 as a catalytic intermediate. After the oxidized E2-bound lipoyl moiety has left its E3 binding site, free lipoate from the medium

Table 1
Changes in the 2-oxoglutarate dehydrogenase complex activities due to the treatment of the complex with dipheniliodonium chloride*

Preparation	Activity			
	Overall (reactions 1–3)	E1	E3	Thioredoxin-insulin reduction
Control	100	100	100	100
Diphenyliodonium chloride-treated complex	37	142	17	24

*For experimental conditions see section 2.

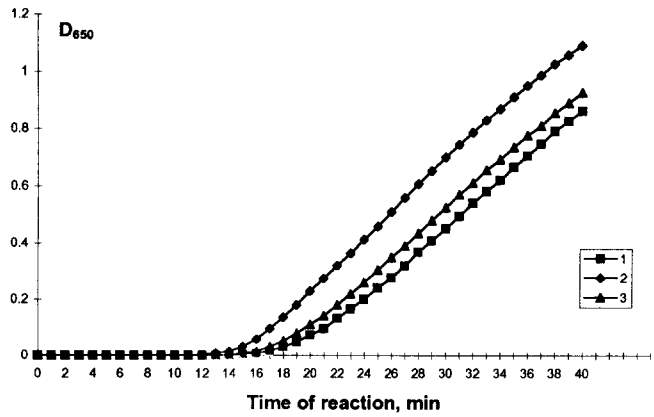

Fig. 2. Insulin reduction by *E. coli* thioredoxin (0.05 mg/ml) dependent on dihydrolipoate enantiomers (0.37 mM). Curve 1, *R*-dihydrolipoate; curve 2, *S*-dihydrolipoate; curve 3, *R,S*-dihydrolipoate.

may interact with the reduced E3 intermediate accepting the reducing equivalents and reoxidizing the enzyme.

Dihydrolipoyl acetyltransferase of the pyruvate dehydrogenase complex shows also stereoselectivity for *R*-lipoate [8,14]. However, this enzyme cannot execute the reduction of lipoate, since the formation of the two electron-reduced enzyme in this case is not possible and each active site of E2 contains only one binding site for the lipoate moiety [15,16]. This argues against the assumption that Reaction 4 may proceed in the active center of E2.

Thus, the lipoate-mediated thioredoxin reduction needs the sequential work of all three catalytic components of the 2-oxoacid dehydrogenase complexes. This supports the possible physiological significance of the functional interplay between the dehydrogenases of 2-oxoacids and mitochondrial thioredoxin, since the thioredoxin may be reduced in mitochondria by the existing catalytic unit for the 2-oxoacid oxidation. With lipoate as the terminal electron acceptor instead of NAD^+, the latter process may be coupled to the pathways utilizing its energy through the reduced thioredoxin. Such a possibility appears even more probable, if we take into account the high protein concentration inside the mitochondria along with the large number of mitochondrial dehydrogenases competing for NAD^+: this makes the fact obvious that in vivo the 2-oxoacid dehydrogenase complexes do not operate under NAD^+-saturated conditions, but are allowed to participate in the reduction of physiologically occuring lipoate.

Mitochondrial thioredoxin reduced at the expense of 2-oxoacid could play an important role in mitochondrial DNA synthesis, viz. by providing reducing equivalents for the formation of 2′-deoxyribonucleotide pools catalyzed by a presumed organelle-located ribonucleotide reductase [17].

The 2-oxoacid-dependent lipoate-reductase activity of the enzyme complexes studied in this work should be taken into account when investigating the lipoate effect upon operating complexes. Such an investigation done with pyruvate dehydrogenase complexes from various sources showed different effects of *R*- and *S*-lipoate [9]. The possibility of the *R*-lipoate reduction by the complexes might be responsible for the observed difference, since the system with the *R*-enantiomer is able to reduce it and will contain therefore both the oxidized and re-

duced forms of lipoate, while in case of the *S*-enantiomer only the oxidized form is present.

Thus, the lipoate reductase activity of the 2-oxoacid dehydrogenase complexes has implications for understanding both the functioning of the complexes and their integration in the different pathways of mitochondrial metabolism.

Acknowledgements: This work was supported by grants from the Volkswagen Foundation, Hannover, and ASTA Medica AG, Frankfurt. The authors also thank Dr. G. Raddatz for valuable discussions.

References

[1] Bunik, V.I. and Follmann, H. (1993) FEBS Lett. 336, 197–200.
[2] Yeaman, S.J. (1989) Biochem. J. 257, 625–632.
[3] Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13965.
[4] Bodenstein-Lang, J., Buch, A. and Follmann, H. (1989) FEBS Lett. 258, 22–26.
[5] Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632.
[6] Williams, C.H. (1977) The Enzymes 13, 89–173.
[7] Koeplin, R. (1988) Ph.D. Thesis, University of Tuebingen.
[8] Yang, Y.-S. and Frey, P.A. (1989) Arch. Biochem. Biophys. 268, 465–474.
[9] Koeplin, R., Ulrich, H. and Bisswanger, H. (1991) in: Biochemistry and Physiology of Thiamin Pyrophosphate Enzymes (Bisswanger, H. and Ullrich, J. Eds.) VSN Weinheim, pp. 242–250.
[10] Stanley, C.J. and Perham, R.N. (1980) Biochem. J. 191, 147–154.
[11] Segal, A.W., West, I., Wientjes, F., Nugent, J.H.A., Chavan, A.J., Haley, B., Gracia, R.C., Rosen, H. and Scarce, G. (1992) Biochem. J. 248, 781–788.
[12] O'Donnell, V.B., Tew, D.G., Jones, O.T.G. and England, P.J. (1993) Biochem. J. 290, 41–49.
[13] Buech, K. (1994) Ph.D. Thesis, University of Tuebingen.
[14] Oehring, R. and Bisswanger, H. (1992) Biol. Chem. Hoppe-Seyler 373, 333–335.
[15] Mattevi, A., Oblomova, G., Kalk, K.H., Teplyakov, A. and Hol, W.G.J. (1993) Biochemistry 32, 3887–3901.
[16] Mattevi, A., Oblomova, G., Kalk, K.H., Kok, A. de and Hol, W.G.J. (1993) J. Mol. Biol. 230, 1183–1199.
[17] Young, P., Leeds, J.M., Slabaugh, M.B. and Mathews, C.K. (1994) Biochem. Biophys. Res. Commun. 203, 46–52.