Volume 336, number 2, 197–200 FEBS 13404 December 1993
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Thioredoxin reduction dependent on α-ketoacid oxidation by α-ketoacid dehydrogenase complexes

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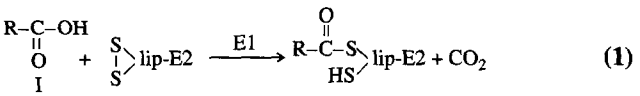
Received 13 September 1993

The pyruvate and α-ketoglutarate dehydrogenase complexes isolated from pig heart mitochondria promote the reduction of thioredoxin in the presence of their α-ketoacid substrates, coenzyme A, and free lipoate. Substrate-specific generation of reduced thioredoxin was established by two independent methods, viz. reduction of insulin and thioredoxin reductase-catalyzed NADPH formation. Dihydrolipoate accumulating in the absence of NAD^+ is the likely intermediate. A redox function in α-ketoacid oxidation provides a potential role for the specific thioredoxins previously identified by us in mitochondria.

Thioredoxin; Thioredoxin reductase; α-Ketoacid dehydrogenase complex; Insulin; Lipoate; Mitochondrion

1. INTRODUCTION

Thioredoxins are small proteins containing a redox-active di-cysteine structure. They are involved in a variety of physiologically important functions by virtue of their ability to serve as hydrogen donors or to reduce disulfide bonds in proteins [1]. Oxidized thioredoxins can be reduced at the expense of NADPH in the reaction catalyzed by thioredoxin reductase [1]. However, they are easily reduced also by dithiols, including dihydrolipoate [2]. The latter is known to be an intermediate in the oxidative decarboxylation of α-ketoacids catalyzed by the α-ketoacid dehydrogenase complexes (equations **1–3**) [3].



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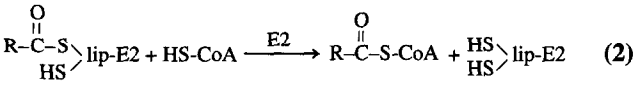

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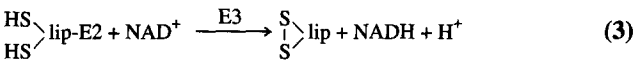

(3)

In reaction **(1)**, the reducing equivalents from an α-ketoacid oxidized by the dehydrogenase complex component E1 are accepted by lipoic acid covalently bound to the second enzyme, dihydrolipoate acyltransferase (E2). Dihydrolipoate is generated after acyl transfer from acyldihydrolipoate to coenzyme A (reaction 2). In the presence of NAD^+ dihydrolipoate is reoxidized by dihydrolipoate dehydrogenase E3 (reaction **(3)**). In the absence of NAD^+, however, the E2-bound dihydrolipoate can be oxidized by other means. We suggested that its reducing equivalents are transferred to the excess of lipoic acid present in solution, either through non-enzymatic thiol-disulfide exchange reactions between enzyme-bound dihydrolipoate and free lipoate, or catalyzed by E3 which, after reduction by enzyme-bound dihydrolipoate, is capable of effectively reducing free lipoate.

Thus, in the presence of lipoate instead of NAD^+, α-ketoacid dehydrogenases may be expected to accumulate dihydrolipoate during the oxidation of their α-ketoacid substrates. As far as we know, there are no data in the literature about this reaction. Only E1-dependent reductive acylation of free lipoate (i.e. reaction **(1)** with free lipoate), and the transacylation reaction between enzyme-bound acyldihydrolipoate and free lipoate have been studied [4]. However, these reactions proceeded only to a very limited extent.

The aim of this work was to study the possibility and efficiency of thioredoxin reduction dependent on the dihydrolipoate formed during α-ketoglutarate or pyruvate oxidation catalyzed by the α-ketoglutarate or pyruvate dehydrogenase complex, respectively. It has been prompted by our earlier observation of specific mitochondrial thioredoxins [5,6], which are in search of a function.

2. MATERIALS AND METHODS

The α-ketoglutarate and pyruvate dehydrogenase complexes were isolated from pig hearts in parallel according to [7], omitting final

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Abbreviations: CoA, coenzyme A; lip, lipoate; KGD, α-ketoglutarate dehydrogenase.

chromatographic separation on Sepharose CL-2B. Triton X-100 was included only during extraction of the complexes from the tissue. The preparation from pig heart did not permit separation of the α-ketoglutarate and pyruvate dehydrogenase complexes by differential polyethylene glycol precipitation as described for the enzymes from bovine heart [7]. The purified fraction containing both complexes was used for thioredoxin reduction.

Thioredoxin reductase was obtained from *E. coli* cells according to [8]. Thioredoxin from *E. coli* (oxidized) was from Calbiochem; coenzymes, insulin, D,L-lipoic acid and dithiothreitol were from Serva; α-ketoglutarate, pyruvate and cysteine from Merck.

Kinetic measurements were done at room temperature in 0.1 M potassium phosphate buffer pH 7.0 with the specified components, using Kontron 930 or Aminco DW 2000 spectrophotometers.

3. RESULTS

Thioredoxin is known to catalyze the reduction of insulin by dithiols [2]. Precipitation of the free B-chains liberated by reduction results in an increase in the turbidity of the reaction mixture measurable at 650 nm. This spectrophotometric assay proved well suited to demonstrate the α-ketoacid-dependent reduction of thioredoxin. α-Ketoglutarate or pyruvate were oxidized by the corresponding enzyme complex in the presence of 0.2–0.8 mM CoA as the acyl residue acceptor and 1–4 mM lipoate as the possible electron acceptor. Only when thioredoxin was present in the reaction medium could insulin reduction be observed (Fig. 1, curves 1 and 2). The reaction was dependent on the thioredoxin concentration as well as on the amount of lipoate and of the enzyme complex (Table I). It was also strictly dependent on the α-ketoacid substrates, indicating that the thiol groups of coenzyme A and of cysteine (required for the reduced state of CoA) present in solution did not contribute to the observed rate of insulin reduction.

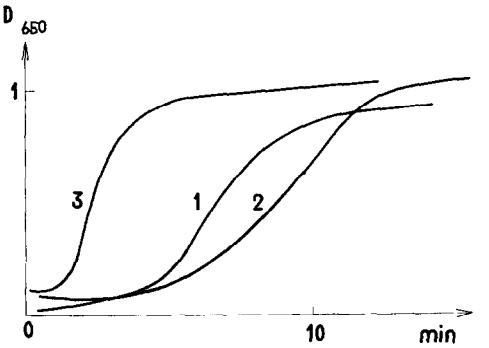

Fig. 1. Reduction of insulin, measured at 650 nm, by *E. coli* thioredoxin coupled to α-ketoacid dehydrogenase complexes from pig heart mitochondria. Curve 1: α-Keto-glutarate dehydrogenase/α-ketoglutarate; curve 2: pyruvate dehydrogenase/pyruvate; curve 3: 1 mM dithiothreitol, no enzyme (control). See Table I for concentrations, other components, and conditions.

Insulin reduction dependent on α-ketoacid oxidation plus thioredoxin was qualitatively comparable to that produced by 1 mM dithiothreitol (Fig. 1, curve (3)). Obviously, the rates of the enzyme-linked reactions were limited by the α-ketoacid dehydrogenase activities,

Table I

Maximum rates of α-ketoacid dehydrogenase- and thioredoxin-dependent insulin reduction[a]

Components of reaction mixture	Thioredoxin ($\mu g \cdot ml^{-1}$)	Other variable component	$\Delta A_{650} \cdot min^{-1}$
Pyruvate dehydrogenase[b], substrate pyruvate (4 mM)			
Complex, 0.24 mg · ml^{-1}	40		0.096
Thiamine-PP, 0.4 mM	80		0.188
Coenzyme A, 0.8 mM			
Lipoate, 4 mM			
$MgCl_2$, 1.2 mM			
α-Ketoglutarate dehydrogenase[b], substrate α-ketoglutarate (2 mM)			
Complex, 0.08 mg · ml^{-1}	20		0.017
Coenzyme A, 0.4 mM	40		0.048
Lipoate, 1.2 mM	80		0.069
	160		0.094
Complex 0.08 mg · ml^{-1}	160	coenzyme A, 0.2 mM	0.055
Lipoate, 1.2 mM	160	0,4	0.094
	160	0.8	0.099
Complex, 0.08 mg · ml^{-1}	160	lipoate, 0.4 mM	0.062
Coenzyme A, 0.8 mM	160	1.2	0.110
	160	4.0	0.139
Coenzyme A, 0.4 mM	160	complex, 0.02 mg · ml^{-1}	0.035
Lipoate, 1.2 mM	160	0.08	0.094

[a] 0.9 mg insulin · ml^{-1} in potassium phosphate buffer, pH 7.0.
[b] Referred to as complex.

in accord with the 1.7-fold lower pyruvate-NAD^+ reductase activity in the enzyme fraction used and the dependence of insulin reduction on the complex concentration (Table I).

It must be noted that the half-maximal rate of the reaction studied was obtained at substrate concentrations (> 0.4 mM α-ketoglutarate and 0.2 mM CoA) higher than the known K_m values of α-ketoglutarate (0.01–0.2 mM) and CoA (0.0001–0.0035 mM) determined for the pig heart KGD complex by different authors [9]. This discrepancy is due to the non-applicability of standard kinetics to the insulin reduction assay. It is impossible to measure the initial rate of α-ketoglutarate dehydrogenase reaction in this system which functions only when a high degree of substrate conversion to the products is reached. The lower substrate concentrations will be depleted before significant product accumulates and, consequently, do not induce measurable insulin precipitation.

In a second approach, α-ketoacid-dependent thioredoxin reduction was proved more directly by coupling the system with thioredoxin reductase. Thioredoxin reductase activity is usually determined in the direction of NADPH oxidation concomitant with thioredoxin reduction. The reverse reaction, thioredoxin oxidation was followed in our experiments which allowed to detect thioredoxin reduced in the course of α-ketoacid oxidation. When $NADP^+$ with thioredoxin reductase were added to a preincubation medium containing oxidized thioredoxin and the KGD complex with α-ketoglutarate, CoA and lipoate, $NADP^+$ reduction was observed (Fig. 2). No reaction occurred in the absence of thioredoxin, or after preincubation of thioredoxin in a reaction medium without α-ketoglutarate. The initial burst in NADPH production in such a system was determined by the accumulation of reduced thioredoxin in the preincubation medium; the burst amplitude depended on the preincubation time and the thioredoxin content (data not shown). It was followed by a slower, linear phase of $NADP^+$ reduction determined by the production of dihydrolipoate in the KGD reaction. The reaction rate in this stationary phase increased proportionally with increasing amounts of KGD complex in the sample (Fig. 2, arrows 2 and 3). After prolonged incubation (more than one hour) the initial amount of enzyme added did no longer contribute much to the reaction rate, most likely because of the known inactivation of KGD complex during the reaction [10].

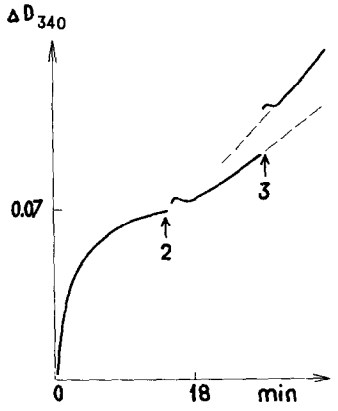

Fig. 2. NADPH production in a reaction mixture containing *E. coli* thioredoxin (73 μg · ml^{-1}), KGD complex (65 μg · mg^{-1}), α-ketoglutarate (3.6 mM), coenzyme A (1.1 mM), cysteine (2 mM), and lipoate (1.8 mM). After a 1 h incubation period, thioredoxin reductase (76 μg · ml^{-1}) and $NADP^+$ (1.6 mM) were added and $NADP^+$ reduction was recorded at 340 nm. Arrows: additional portions of KGD complex (equal to the first amount of enzyme) were added at the indicated times.

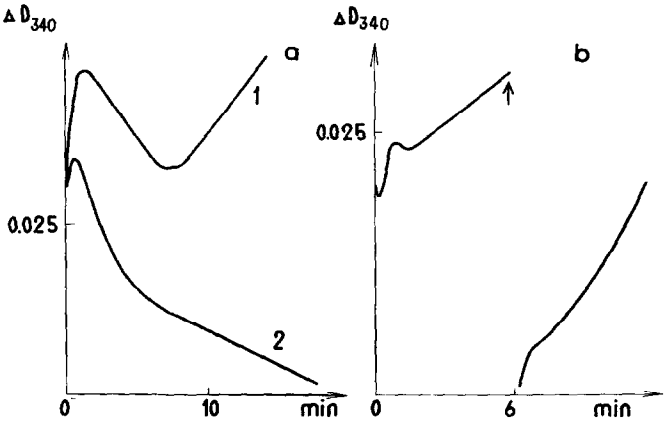

Fig. 3. NADPH production in reaction mixtures containing $NADP^+$ thioredoxin reductase, thioredoxin, and the α-ketoglutarate dehydrogenase complex without a preincubation period. (a) Complete system containing KGD (60 μg · ml^{-1}), α-ketoglutarate (3.3 mM), coenzyme A (0.7 mM), cysteine (1.4 mM), lipoate (1.7 mM), $NADP^+$ (1.7 mM), thioredoxin reductase (80 μg · ml^{-1}) plus thioredoxin (33 μg · ml^{-1}) (curve 1) or without thioredoxin (control, curve 2). (b) Difference spectroscopy measurements with a sample cuvette containing the complete system as above and a reference cuvette containing the same mixture without thioredoxin-thioredoxin reductase. The reaction was started by simultaneous addition of KGD to both cuvettes. Arrow: addition of a second, identical quantity of KGD complex.

To couple thioredoxin reductase and KGD-promoted dihydrolipoate formation, NADPH production was also initiated by addition of the KGD complex to the complete system containing α-ketoglutarate, CoA, lipoate, oxidized thioredoxin, thioredoxin reductase, and $NADP^+$. Complex spectral changes at 340 nm were observed in this variant (Fig. 3) which can in part be attributed to the KGD reaction alone (Fig. 3a, control curve without thioredoxin). The rate of dihydrolipoate-thioredoxin-dependent NADPH production was better defined in difference measurements using the KGD-thioredoxin systems combined in the sample and only the KGD system in a reference cuvette (Fig. 3b). These values, then, became comparable to those in the stationary phase of the experiments including a preincubation period (Fig. 2). Again, as in the case of insulin reduction, the reaction was dependent on the amount of KGD complex (Fig. 4) and far higher activities were

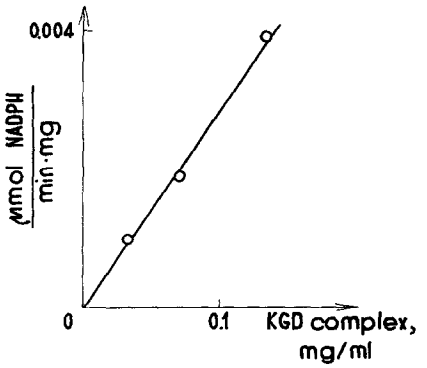

Fig. 4. Dependence of thioredoxin reductase activity (NADPH production), in the presence of *E. coli* thioredoxin, on the concentration of α-ketoglutarate dehydrogenase complex. Conditions and reaction medium as in Fig. 3b.

induced by 1 mM dithiothreitol, confirming the limiting role of KGD-catalyzed reactions in thioredoxin reduction.

4. DISCUSSION

The possibility of thioredoxin reduction dependent on α-ketoglutarate or pyruvate oxidation in the presence of coenzyme A and free lipoate has been explored in the present work. Effective reduction of oxidized thioredoxin present in such a medium could be established by two independent methods, utilizing the nascent reduced thioredoxin for reduction of insulin, or as a substrate of NADP-thioredoxin reductase. Obviously, the observed reduction of thioredoxin is based on the generation of dihydrolipoate by the α-ketoacid dehydrogenase complexes catalyzing oxidative decarboxylation in the absence of NAD^+ with coenzyme A as acyl and excess free lipoate as electron acceptor. No other compound capable of reducing the disulfide bridge in an oxidized thioredoxin but reduced lipoate has been present in these reaction media.

There are two aspects of interest in this reaction. On the one hand, lipoic acid is a pharmacological agent with many biological effects [11]. It was shown that in some cases the disulfide of lipoate exerts an effect opposite to the action of glutathione disulfide [12]. This should reflect a special way of lipoate reduction independent of other cellular disulfides. α-Ketoacid-dependent reduction could be such a way. The possibility of transfer of reducing equivalents from dihydrolipoate to thioredoxin must be kept in mind in studying the biological effects of lipoic acid because of the multiple biochemical functions of reduced thioredoxin.

On the other hand, the reaction is of major interest in connection with the existence of specific mitochondrial thioredoxins [5,6]. We suggest that α-ketoacid-dependent thioredoxin reduction is an integral part of the thioredoxin cycle in mitochondria. The use of *E. coli* thioredoxin, which is available in substrate quantities, in our present work is warranted in view of the unspecificity of the bacterial protein in vitro and its similarity to the mitochondrial, but not the cytoplasmic mammalian thioredoxin. More physiological conclusions, however, will have to await experiments combining mitochondrial thioredoxins and enzyme complexes from the same source.

Acknowledgements: We thank H. Braun and A. Lichter for their skillful assistance. This study has been made possible by a travel grant from Deutsche Forschungs-gemeinschaft (to V.B.), and was supported by Fonds der Chemischen Industrie.

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