ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 8 (2000) 165–174

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Increased catalytic performance of the 2-oxoacid dehydrogenase complexes in the presence of thioredoxin, a thiol–disulfide oxidoreductase

Victoria Bunik *

A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation

Received 5 December 1998; accepted 9 April 1999

Abstract

Bacterial and mammalian pyruvate and 2-oxoglutarate dehydrogenase complexes undergo an irreversible inactivation upon accumulation of the dihydrolipoate intermediate. The first component of the complexes, 2-oxoacid dehydrogenase, is affected. Addition of thioredoxin protects from this inactivation, increasing catalytic rates and limiting degrees of the substrate transformation to products, acyl-CoA and NADH. Although the redox active cysteines of thioredoxin are essential for its interplay with the complexes, the effects are observed with both dithiol and disulfide forms of the protein. This indicates that thioredoxin affects an SH/S–S component of the system, which is present in the two redox states. The complex-bound lipoate is concluded to be the thioredoxin target, since (i) both dithiol and disulfide forms of the residue are available during the catalytic cycle and (ii) the thioredoxin reaction with the essential SH/S–S group of the terminal component of the complex, dihydrolipoyl dehydrogenase, is excluded. Thus, the thioredoxin disulfide interacts with the dihydrolipoate intermediate, while the thioredoxin dithiol reacts with the lipoate disulfide. Kinetic consequences of such interplay are consistent with the observed thioredoxin effects. Owing to the essential reactivity of the SH/S–S couple in thioredoxin, the thiol–disulfide exchange between thioredoxin and the lipoate residue is easy reversible, providing both protection (by the mixed disulfide formation) and catalysis (by the appropriate lipoate release). In contrast, non-protein SH/S–S compounds prevent the inactivatory action of dihydrolipoate intermediate only at a high excess over the complex-bound lipoate. This interferes with the catalysis-required release of the residue from its mixed disulfide. Therefore, only thioredoxin is capable to 'buffer' the steady-state concentration of the reactive dithiol. Such action represents a new thioredoxin function, which may be exploited to protect other enzymes with exposed redox-active thiol intermediates.

Keywords: 2-Oxoacid dehydrogenase complex; Catalysis-associated inactivation; Thioredoxin; Lipoate; Cysteine; Glutathione

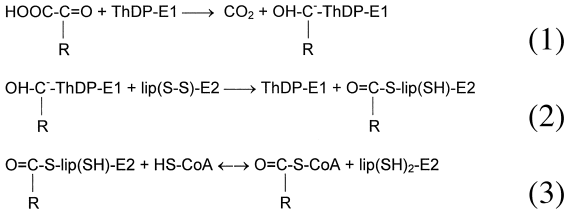
Abbreviations: E1, 2-oxoacid dehydrogenase; E1p, pyruvate dehydrogenase; E1o, 2-oxoglutarate dehydrogenase; E2, dihydrolipoate acyltransferase; E3, dihydrolipoate dehydrogenase; ThDP, thiamin diphosphate; lip, lipoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; v, reaction velocity in the absence of effectors; v_a, reaction velocity affected by an effector; k'_i, apparent rate constant of the catalysis-induced inactivation

* Tel.: +7-095-939-14-56; Fax: +7-095-939-31-81; E-mail: vbun@bac.genebee.msu.su

1381-1177/00/$ - see front matter
PII: S1381-1177(99)00054-5

1. Introduction

2-Oxoacid dehydrogenase complexes catalyze an irreversible oxidation of 2-oxoacids, producing acyl-CoA's and NADH via reactions 1–5:



(1)

(2)

(3)

$$\mathrm{lip(SH)_2 - E2 + E3(S-S) - FAD} \leftarrow\rightarrow \mathrm{lip(S-S) - E2 + E3(SH)_2 - FAD} \quad (4)$$

$$\mathrm{E3(SH)_2 - FAD + NAD^+} \leftarrow\rightarrow \mathrm{E3(S-S) - FAD + NADH + H^+} \quad (5)$$

The complexes are highly organized systems [1,2] including multiple copies of at least three different enzymes. These are the substrate-specific and thiamine diphosphate (ThDP)-dependent 2-oxoacid dehydrogenases (E1), in particular, pyruvate dehydrogenase (E1p, R = CH_3–) and 2-oxoglutarate dehydrogenase (E1o, R = COOH–$(CH_2)_2$–); dihydrolipoyl acyltransferase (E2) with the covalently bound lipoate residue (lip); and the FAD-containing dihydrolipoyl dehydrogenase (E3).

Recently we have found that the activity of the 2-oxoacid dehydrogenase complexes rapidly decreases during reaction at low NAD^+ concentrations [3]. Under the same conditions the complexes were activated by some thioredoxins [3,4]. The latter are small (11–12 kDa) thermostable proteins with a disulfide bridge in the active site [5,6], responsible for the thiol–disulfide oxidoreductase activity of thioredoxins. Extremely high reactivity of the essential dithiol–disulfide couple enables thioredoxins to reduce disulfides in proteins [7,8], to activate target proteins with redox-active thiol groups [9,10], to mediate redox-regulation of transcription factors [11,12] and cytokine synthesis [13], to promote cell growth [14,15], to scavenge free radicals [16–18]. Although the highest efficiency is usually achieved with the physiological targets of thioredoxins, they often exhibit a significant level of cross-reactivity. This allows one to use a well-characterized and commercially available protein from *Escherichia coli* for model studies in different systems. In the present work this protein was employed to investigate the mechanism of the thioredoxin stimulation of the 2-oxoacid dehydrogenase complexes from different sources. The results obtained show that thioredoxin may regulate the flux through the dehydrogenase complexes, supporting an optimal steady-state concentration of the complex-bound dihydrolipoate. This protects the complexes from the catalysis-associated inactivation which is caused by accumulation of the dihydrolipoate intermediate. As a result, an increased catalytic performance is observed with thioredoxin in a broad interval of conditions, including initially low NAD^+ concentrations and prolonged reaction times leading to NAD^+ depletion.

2. Experimental

2.1. Materials

Coenzyme A, insulin, DTNB were obtained from Sigma; ThDP, glutathione disulfide, glutathione from Serva; 2-oxoglutarate, pyruvate, cysteine, cystine from Merck; NAD^+, DTT from Boehringer; thioredoxin of *E. coli* from Calbiochem. Bacterial 2-oxoacid dehydrogenase complexes were gifts from Dr. Hans Bisswanger (Tübingen University, Germany) and Dr. Aart DeKok (Agricultural University of Wageningen, The Netherlands).

2.2. Enzyme isolation and assays

2-Oxoglutarate and pyruvate dehydrogenase complexes from pig heart were isolated according to Ref. [19] with modifications given in Ref. [20] and stored frozen in 0.1 M potassium phosphate, pH 7.0, at -20°C.

The 2-oxoglutarate and pyruvate dehydrogenase complexes were assayed in 0.1 M potassium phosphate, pH 7.0 and 7.6, respectively, containing 1 mM ThDP and 1 mM magnesium chloride. Saturating concentrations of substrates were 2 mM 2-oxoacid, 0.05 mM CoA, 2.5 mM NAD^+. Assay of the complexes preincubated with their substrates was started by addition of 2.5 mM NAD^+. The $p(t)$ curves were followed during 4 min. No significant lag-phase was observed, indicating that the inactivation determined from initial velocities was not reversed in the medium with high NAD^+ concentration. The E1 activity was assayed by the ferricyanide reduction as in Ref. [21]. The E3 activity was determined by the dihydrolipoamide-dependent NAD^+ reduction in 0.1 M potassium phosphate, pH 7.6, at 4 mM dihydrolipoamide and 1 mM NAD^+. If not otherwise indicated, the disulfide form of thioredoxin was used. Substrate excess was removed from the inactivated enzyme by gel-chromatography on HiTrap™ 5 ml desalting column (Pharmacia), eluted with 0.1 M potassium phosphate, pH 7.0, at 4 ml min^{-1} at room temperature.

2.3. Reduction of thioredoxin

Thioredoxin (2 mg ml^{-1}) was incubated with DTT (10 mM) for 40 min at 4°C in 0.1 M potassium phosphate, pH 7.0. Reduced thioredoxin was separated from the DTT excess using a HiTrap™ column as described above. The treatment resulted in appearance of the two DTNB-reactive SH groups per thioredoxin molecule. The thiols were resistant to air oxidation during the time of experiment (an hour), when thioredoxin was kept on ice in 0.1 M potassium phosphate, pH 7.0.

2.4. Analytical methods

Thiol groups of thioredoxin (2–3 μM) were titrated with DTNB as described in Ref. [3]. The thioredoxin concentration was determined at 280 nm applying molar absorbance coefficient 13 700 M^{-1} cm^{-1} [7].

The level of CoA reduction was controlled in separate titration experiments with DTNB, or enzymatically from the maximum NAD^+ reduction (reactions 1–5) with limiting CoA and an excess of 2-oxoglutarate dehydrogenase complex and other substrates. The CoA preparations contained more than 95% of reduced CoA.

Kinetic measurements were carried out at 25°C on a Uvicon spectrophotometer (Kontron Instruments, Neufahrn, Germany) with a 5 s delay after starting the reaction. The product accumulation curves were analyzed by a modification of Huggenheim's method as described in Ref. [21]. The apparent inactivation rate constant, k'_i, was determined from a slope of a semilogarithmic plot $\ln(\Delta A_{340,i}) = f[\Delta t(i-1)]$ at $\Delta t = 30$ s, where i is a number of the consecutive time intervals Δt, in which $p(t)$ curve is divided. The slopes of the least-squares regression lines for the data points were computed using MathCAD 2.09 (MathSoft).

Spectra were measured in 0.1 M potassium phosphate, pH 7.0 under aerobic conditions. Substrates were added both to the reference and sample cells, with the enzyme present in the sample cell.

3. Results and discussion

3.1. Inactivation of the 2-oxoacid dehydrogenase complexes during catalysis

At saturating 2-oxoacid, CoA and low NAD^+ concentrations, the rate of the 2-oxoacid dehydrogenase reaction decreased with time so that practically no activity was left after several minutes of the reaction. This was observed with both bacterial and mammalian pyruvate and 2-

oxoglutarate dehydrogenase complexes. The decrease was not caused by NAD^+ depletion, since (i) a new NAD^+ addition did not restore the initial velocity and (ii) only a fraction of NAD^+ was reduced before the reaction practically stopped (Table 1). The limiting degree of the substrate conversion depended on the quantity of added enzyme. At 0.018 mg ml^{-1} of 2-oxoglutarate dehydrogenase complex the NAD^+ conversion was 3-fold higher than in the probe with 0.002 mg ml^{-1} enzyme (Table 1). These data point to an irreversible inactivation of the catalyst in the course of reaction [22].

Preincubation of the enzyme complexes with the components of the reaction mixture showed that it is the combination of 2-oxoacid and CoA that causes the loss of activity (Fig. 1, curve 1). Neither 2-oxoacid nor CoA alone induced the inactivation (curves 2,3). Moreover, in the presence of 2-oxoacid, the inactivation becomes more pronounced with increasing the enzyme saturation by CoA. While 40% of CoA was converted to product at 2 μM CoA, only 15% of CoA could be utilized in the reaction at 50 μM CoA (Table 2).

According to the mechanism of the 2-oxoacid oxidation (reactions 1–5), the complex-bound dihydrolipoate is expected when the complexes are exposed to 2-oxoacid and CoA (reactions 1–3). Formation of the dihydrolipoate intermediate was supported by spectral changes in the absorption of the complex-bound flavin coenzyme. The spectrum was unchanged in the pres-

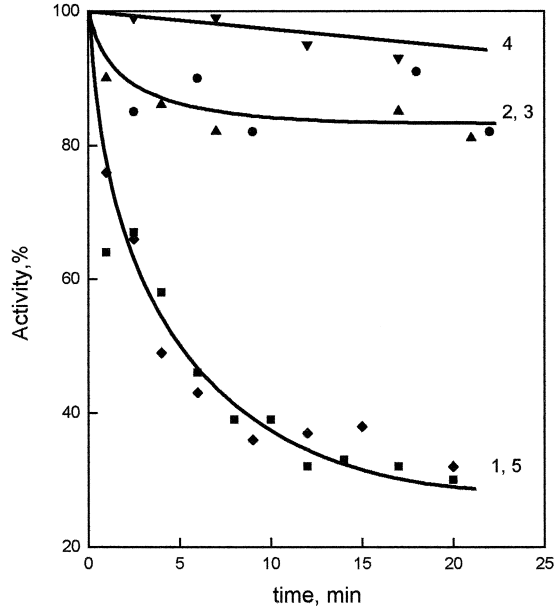


Fig. 1. Activities of the pig heart 2-oxoglutarate dehydrogenase complex and its E1 and E3 components during preincubation of the complex (0.3 mg ml^{-1}) in the reaction medium with saturating concentrations of substrates. (1)—The overall activity, preincubation with both 2-oxoglutarate and CoA; (2)—the overall activity, preincubation with CoA; (3)—the overall activity, preincubation with 2-oxoglutarate; (4)—the E3 activity, preincubation with both 2-oxoglutarate and CoA; (5)—the E1 activity, preincubation with both 2-oxoglutarate and CoA.

Table 1
Percentage of NAD^+ reduced in the 2-oxoacid dehydrogenase reactions at saturating 2-oxoacids, CoA and low NAD^+ concentrations

2-Oxoacid dehydrogenase complex	Complex concentration, mg ml^{-1}	NAD^+ reduced, %[a]	
		Without thioredoxin	With thioredoxin
2-Oxoglutarate dehydrogenase (pig heart)	0.018	42 ± 2	57 ± 4
	0.002	13 ± 1	43 ± 4
2-Oxoglutarate dehydrogenase (*A. vinelandii*)	0.006	18 ± 1	33 ± 1
Pyruvate dehydrogenase (pig heart)	0.014	25 ± 5	49 ± 4
Pyruvate dehydrogenase (*E. coli*)	0.005	6 ± 2	11 ± 3

[a]The values were determined for the reactions with 16 μM NAD^+, followed during 4 min. In the medium without thioredoxin the reactions were practically completed to this time and the values given thus represent the limiting degrees of the NAD^+ conversion under these conditions. When added, 6.8 μM thioredoxin was used with the pyruvate dehydrogenase complex from *E. coli* and 3 μM thioredoxin in other cases.

Table 2
Limiting degrees of CoA conversion in the 2-oxoglutarate dehydrogenase reaction at variable CoA concentration

CoA added, μM	CoA converted, %[a]	
	Without thioredoxin	With thioredoxin
2	40±7	53±6
5	38±5	70±5
50	15±2	55±1

[a]The values were determined from the final product concentration reached in the reactions catalyzed by 0.003 mg ml^{-1} pig heart enzyme complex at 2 mM 2-oxoglutarate, 0.1 mM NAD^+ and 4.8 μM thioredoxin (when added).

ence of 2-oxoglutarate, but following CoA addition induced a rapid decrease in absorbance at 450 nm with the concomitant increase at 400 nm (not shown). The observed spectral changes are known to proceed under reduction of E3 with dihydrolipoate; additional smoothing of the maximum and its shift to a shorter wavelength are inherent in the $4e^-$-reduced enzyme, where both the catalytically active disulfide and flavin have accepted electrons from dihydrolipoate [23,24]. Since $4e^-$-reduction ('over-reduction') inhibits E3 already at the pre-steady-state and this inhibition is less pronounced with increasing NAD^+ [23–26], the over-reduction could explain the decrease in the overall activity at low (Table 1) or no (Fig. 1) NAD^+. However, unlike the inhibition due to E3 over-reduction, the 2-oxoacid plus CoA-dependent inactivation was not reversed by NAD^+. Measured at 2.5 mM NAD^+ or after preincubation with 1 mM NAD^+, the catalytic rate of the inactivated complex did not reach the level inherent in the non-treated enzyme. Besides, no change in the partial E3 activity was observed during the complex preincubation with 2-oxoglutarate and CoA (Fig. 1, curve 4). In contrast, the inactivation kinetics of the overall (Fig. 1, curve 1) and E1-catalyzed (Fig. 1, curve 5) reactions coincided. This indicates that the first component of the complex, 2-oxoacid dehydrogenase, is responsible for the overall activity decay. No recovery in the activity was observed after liberation of the complex from an excess of substrates by gel-filtration. Following incubation in 0.1 M potassium phosphate, pH 7.0, even supplied with DTT (17 mM), did not restore any activity either.

Thus, an irreversible loss of the 2-oxoacid dehydrogenase complex activity owing to the inactivation of its first component takes place upon formation of the complex-bound dihydrolipoate in the medium with 2-oxoacid and CoA. Effective reoxidation of dihydrolipoate by NAD^+ prevents the 2-oxoacid plus CoA-induced inactivation. This is obvious from the decrease in the apparent rate constant of inactivation, k'_i, at higher NAD^+ concentrations (Fig. 2) and an increase in the limiting degrees of the substrate conversion with increasing NAD^+ (Table 3).

3.2. Alleviation of the catalysis-associated inactivation by thioredoxin

The 2-oxoacid dehydrogenase complexes are protected from the inactivation during catalysis

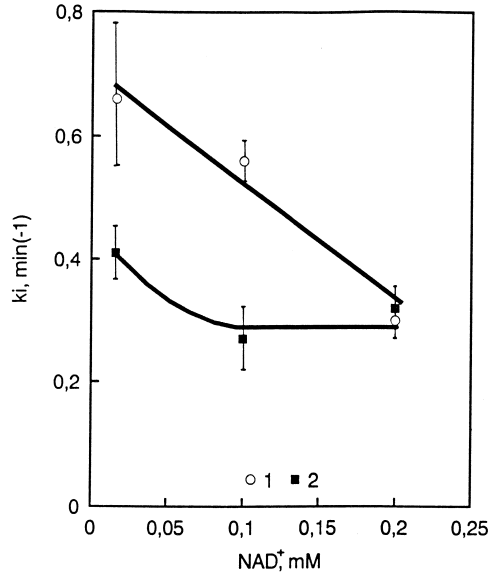


Fig. 2. Influence of *E. coli* thioredoxin on the apparent rate constant of the catalysis-induced inactivation, k'_i, of the *E. coli* pyruvate dehydrogenase complex at different NAD^+ concentrations. The $p(t)$ curves were registered at the complex concentration of 0.005 mg ml^{-1} and saturating concentrations of pyruvate and CoA without (1) and with (2) 4.8 μM thioredoxin.

Table 3
Limiting degrees of the substrate conversion in the 2-oxoglutarate dehydrogenase reaction at variable NAD^+ and thioredoxin concentrations

NAD^+, μM	Substrate conversion[a] (%) at thioredoxin concentrations, μM			
	0	1.6	4.8	13.7
5	5±4	43±4	55±3	62±3
16	12±3	40±3	55±3	60±3
100	15±2	58±2	55±1	68±1
200	44±3	84±2	100±3	n.d.

[a]The percentage of the NAD^+ (at 5, 16 μM NAD^+) and CoA (at 100, 200 μM NAD^+) conversion in the reactions catalyzed by the pig heart enzyme complex (0.003 mg ml^{-1}) at 2 mM 2-oxoglutarate and 50 μM CoA is given.

not only by NAD^+, but also by thioredoxin. This is seen from (i) higher degrees of the NAD^+ and CoA conversion (Tables 1–3), (ii) a decrease in the apparent rate constant of inactivation, k'_i (Fig. 2) and (iii) no activity loss after preincubation with 2-oxoacid and CoA in the presence of thioredoxin (Table 4).

The protection achieved was proportional to the saturation of the complex with thioredoxin. At a fixed concentration of thioredoxin (3 μM), the 3.3-fold increase in NADH production was observed with 0.002 mg ml^{-1} 2-oxoglutarate dehydrogenase complex, while it was only 1.4-fold at the complex concentration of 0.018 mg ml^{-1} (Table 1). At a fixed concentration of the complex (0.003 mg ml^{-1}), the limiting degrees of the CoA and NAD^+ conversion were increased with increasing the thioredoxin concentration (Table 3).

3.3. Effects of the thiol and disulfide forms of thioredoxin

Fig. 2 and Table 3 show that the thioredoxin and NAD^+ effects are nonadditive. The decrease in k'_i to approx. 0.3 min^{-1} brought about by a higher NAD^+ was not further influenced by the thioredoxin concentration effective at a lower NAD^+ (Fig. 2). The substrate conversion is increased by thioredoxin about 10-fold at 5 μM NAD^+, while only a 2-fold increase is observed at 200 μM NAD^+ (Table 3). This suggested that the thioredoxin alleviation of the 2-oxoacid plus CoA-induced inactivation could be caused by the reduction of thioredoxin disulfide at the expense of the dihydrolipoate intermediate, equivalent to the dihydrolipoate-dependent reduction of NAD^+. To check whether such an electron flow per se might be responsible for the thioredoxin action, the influence of the pre-reduced thioredoxin was compared with the effect of its disulfide form. Tables 4 and 5 show that both disulfide and dithiol forms of thioredoxin protect from the substrate-induced inactivation and activate the catalysis. Hence, the thioredoxin action is not due to the dihydrolipoate oxidation by the thioredoxin disulfide.

Table 4
Influence of thiols and disulfides on the inactivation of the pig heart 2-oxoglutarate dehydrogenase complex by 2-oxoglutarate plus CoA

Additions		Concentration, μM	Inactivation[a] $1-v_a/v$, %
None		–	60±10
Disulfides	thioredoxin	3.5	0
	glutathione	20	50±10
		400	0
Thiols	thioredoxin	8	0
	glutathione	20	35±5
		800	0
	cysteine	20	30±5
		800	0

[a]The velocities were measured after 4 min preincubation as described in Section 2.

Table 5
Influence of thiols and disulfides on the rate of the 2-oxoglutarate oxidation at low NAD^+ concentrations

Additions		Concentration, μM	v_a/v[a], %
Disulfides	thioredoxin	5.6	360±40
	glutathione	40	150±20
		400	150±20
	cystine	40	90±20
		400	60±10
Thiols	thioredoxin	5.6	330±30
	glutathione	40	70±10
		400	250±30
	cysteine	40	240±30
		400	340±40

[a]The velocities were measured at the second minute of reaction with 5 μM NAD^+ and 0.001 mg ml^{-1} pig heart enzyme complex.

Given that the thioredoxin redox-active cysteines are essential for its stimulatory effect [3], the similar action of the two redox forms allows one to conclude that the reaction mixture contains both thiol and disulfide of the thioredoxin target (Fig. 3). Under conditions employed, only the complex-bound lipoate and catalytically active disulfide group of E3 may be such targets, since both shuttle between the dithiol and disulfide forms during catalysis (reactions 2–5). The thioredoxin reaction with the catalytically essential cysteines of E3 may be excluded. This follows from the observation that thioredoxin activates the catalysis when E3 limits the reaction rate (at low NAD^+), while thioredoxin interaction with the catalytic groups of limiting enzyme would be inhibitory. In contrast, owing to the excessive formation of the dihydrolipoate intermediate, its reversible thiol–disulfide exchange with thioredoxin should not inhibit catalysis (Fig. 3). Hence, kinetic data point to the complex-bound lipoate residue as the thioredoxin target. This is further supported by available structural information. The active site disulfide of E3 is buried at the end of the narrow channel for the lipoyllysine residue [24,27,28]. This makes the disulfide/dithiol group of E3 inaccessible for thioredoxin. Contrary, the lipoyllysine 'arm' of E2 is known to protrude from the complex structure [1,2] and

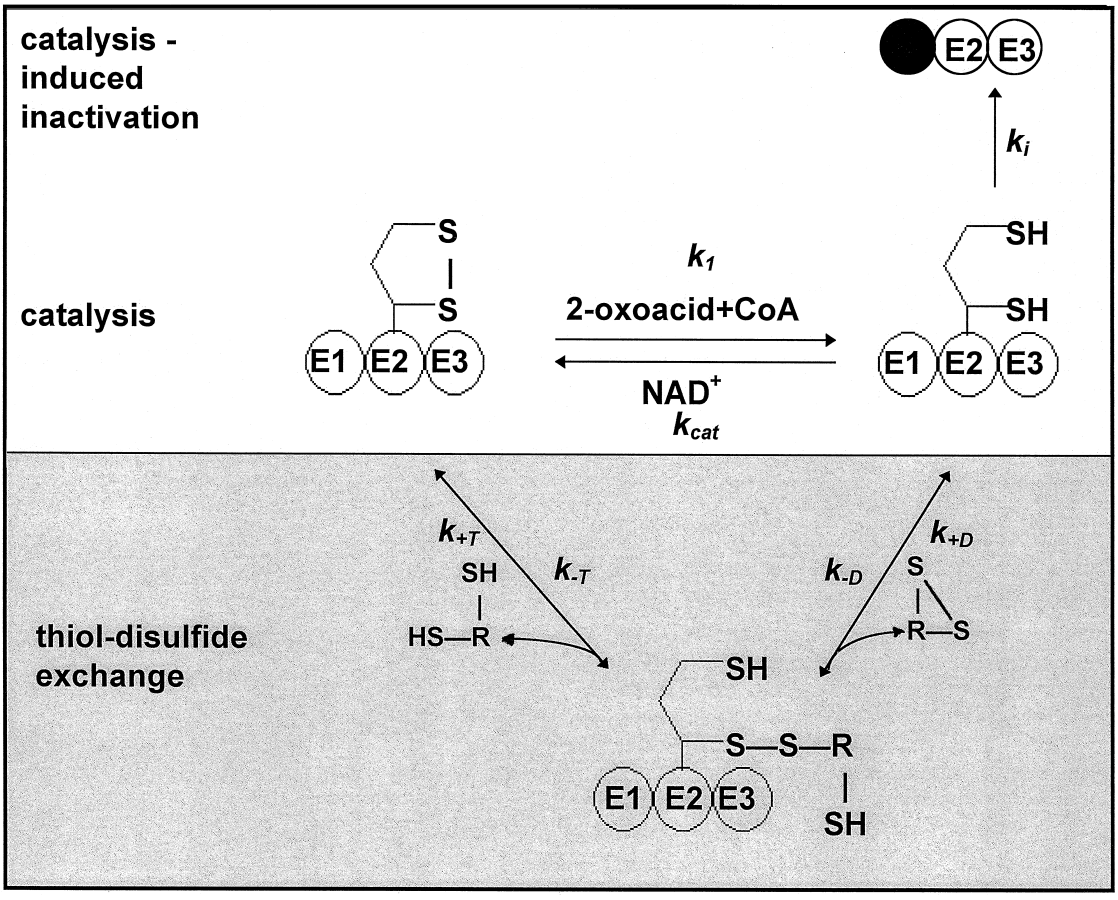


Fig. 3. Proposed mechanism of the interplay between the 2-oxoacid dehydrogenase complexes and thioredoxin. The thioredoxin dithiol ($R-(SH)_2$) and disulfide ($R-S_2$) may be substituted for low molecular weight dithiols and disulfides. The active enzyme (referred as E_a in the text) is shown as a complex of its three components. The inactive enzyme (referred as E_i) contains the inactive E1 component shown in black. The E2-bound lipoate residue forms a mixed disulfide with an external thiol or disulfide (referred as E_{mix}).

therefore should have less steric restrictions for interaction with external compounds.

Thus, in the course of catalysis at low NAD^+ (reactions 1–5) or during preincubation with 2-oxoacid and CoA (reactions 1–3), thioredoxin disulfide interacts with dihydrolipoate, while thioredoxin dithiol reacts with lipoate (Fig. 3). In the former case, thioredoxin competes with the irreversible inactivation induced by the dihydrolipoate excess. In the latter case thioredoxin slows down the reductive acylation of the complex-bound disulfide, ES_2, (reaction 2), eventually leading to the accumulation of the complex-bound dihydrolipoate, $E(SH)_2$, and inactivation. Analysis of Eqs. (6)–(9) which describe the concentrations of different enzyme species and the rates of the catalysis-induced inactivation, v_i, and catalysis, v_{cat}, in the proposed mechanism indicates that the mechanism is fully consistent with the observed thioredoxin effects. [1]

$$E_{total} = [E_a]_t + [E_i]_t \quad (6)$$

$$[E_a]_t = [ES_2]_t + [E(SH)_2]_t + [E_{mix}]_t \quad (7)$$

$$v_{i,t} = k_i[E(SH)_2]_t \quad (8)$$

$$v_{cat,t} = k_{cat}[NAD^+][E(SH)_2]_t \quad (9)$$

The flow from the active enzyme complex, E_a, to that inactive, E_i, depends on the current concentration of $E(SH)_2$ (Eq. (8)). In the absence of the thiol–disulfide equilibrium with thioredoxin ($E_{mix} = 0$), only the catalytic process competes with the inactivation (Fig. 3). Hence, the observed rate of the enzyme transition to the inactive form, E_i, is proportional to the partition ratio $v_i/v_{cat} = k_i/(k_{cat}[NAD^+])$. This ratio shows that increasing NAD^+ slows down the inactivation, that is indeed observed as a decrease in the apparent inactivation rate constant, k'_i, at higher NAD^+ concentrations (Fig. 2). When the complex-bound lipoate participates in the thiol–disulfide exchange with thioredoxin, the active enzyme is distributed between more interconvertible species ($E_{mix} \neq 0$, Eq. (7)). This decreases concentration of $E(SH)_2$. Hence, v_i is decreased as well (Eq. (8)). As a result, a lower k'_i is observed in the presence of thioredoxin (Fig. 2). The mechanism proposed (Fig. 3) shows that both NAD^+ and thioredoxin protect from the irreversible inactivation by reducing concentration of the same intermediate, $E(SH)_2$. This results in the non-additivity ('competition') of the thioredoxin- and NAD^+-afforded protection (Table 3, Fig. 2).

Thus, independent of whether the thioredoxin thiol or disulfide are added, formation of the mixed disulfide with the complex-bound lipoate, E_{mix}, takes place. This scavenges the dihydrolipoate excess and therefore protects from the dihydrolipoate-induced inactivation. At the same time, an efficient catalysis in the presence of thioredoxin implies that formation of E_{mix} is easy reversible. The easy reversibility of the thiol–disulfide exchange reaction is favoured when concentrations of the reactants are close to those at equilibrium. The known difference between the standard redox potentials of the *E. coli* thioredoxin and free lipoate (0.02 V) [29] and the used interval of the activating concentrations of thioredoxin (about 10-fold excess over the complex-bound lipoate) show that under conditions employed the thiol–disulfide exchange is close to equilibrium indeed. Owing to this, E_{mix} with thioredoxin becomes a depot of the catalytically competent enzyme, protected from the catalysis-induced inactivation.

3.4. Influence of other SH / –S–S– compounds

Similarly to thioredoxin, thiols and disulfides of low molecular weight protect the complexes from the catalysis-induced inactivation, but their efficiency is about two orders of magnitude lower than that of thioredoxin (Table 4). Besides, the compounds differ in the magnitude and direction of their influence on activity at

[1] Owing to the catalysis-induced inactivation, the equations include variables dependent on the reaction time, *t*.

low NAD^+. [2] Although the full protection by 0.4 mM glutathione disulfide (Table 4) is observed, it activates the 2-oxoglutarate dehydrogenase reaction only by 50% in comparison to more than 200% activation by the thioredoxin disulfide (Table 5). The inhibition by cystine (Table 5) suggests that glutathione disulfide may partially inhibit the reaction too. Apparently, this effect decreases the activation by glutathione disulfide as compared to thioredoxin. The inhibitory action of disulfides shows that the protection per se does not necessarily result in the activation of catalysis at low NAD^+. This is in accordance with the mechanism proposed (Fig. 3). At a high excess of a disulfide the backward reaction of the dihydrolipoate release is not favoured and therefore the mixed disulfide releases ES_2. Analogous reoxidation of free dihydrolipoyllysine by cystine and glutathione disulfide was observed in the model system [32] under similar conditions (1000-fold excess of disulfides, pH 6.8). Such flow of reducing equivalents to disulfides is competitive to the NAD^+ reduction and therefore inhibits the catalysis.

While cystine is a stronger inhibitor than glutathione disulfide, cysteine is a better activator of catalysis than glutathione (Table 5). This observation is in good agreement with thermodynamics of the free lipoate regeneration from the corresponding E_{mix}. The difference in the standard redox potentials between the dihydrolipoate/lipoate and cysteine/cystine or glutathione/glutathione disulfide couples (0.06 V and 0.03 V, respectively [29]) points to a less stable mixed disulfide of lipoate with cysteine and a favoured dihydrolipoate oxidation by cystine. Thus, our findings indicate that the direction and amplitude of the effect of the SH/S–S compounds on catalysis are determined by reversibility of the thiol–disulfide exchange reactions between the compounds and 2-oxoacid dehydrogenase complexes. Unlike thioredoxin, non-protein thiols and disulfides are able to react with the complex-bound lipoate only at high concentrations. This interferes with the appropriate lipoate release and may inhibit catalysis.

4. Concluding remarks

Formation of the catalytically incompetent dihydrolipoyl dehydrogenase (E3) component upon over-reduction of the complexes with NADH is known to provide the in vivo sensitivity of 2-oxoacid dehydrogenase complexes to the inhibition by high $NADH/NAD^+$ ratio [33]. The present work shows that the complexes are inactivated also under over-reduction with 2-oxoacid and CoA. In this case an irreversible loss of the E1 activity is observed upon dihydrolipoate accumulation, representing another way of the self-regulation of the complexes by reactive intermediates. The steady-state concentration of the dihydrolipoate intermediate may be controlled by the thiol–disulfide exchange with external SH/S–S compounds, resulting in protection of the complexes from the 2-oxoacid, CoA-induced inactivation. Such regulation may tune the function of the complexes in response to the thiol–disulfide status of the surrounding medium.

The thioredoxin interaction with the 2-oxoacid dehydrogenase complexes points to a new function of thioredoxin as a buffer for reactive dithiol intermediates. This action may be used to protect other enzymes with the exposed catalytically active disulfide groups.

[2] In spite of the high concentrations of cystine and glutathione disulfide in the medium (10^{-3}–10^{-4} M), the observed effects of disulfides cannot result from their reaction with a substrate, CoA. No significant thiol–disulfide exchange between these compounds and CoA may be expected within several minutes of assay at pH 7.0 or 7.6, because the reaction of CoA with glutathione disulfide is known to attain equilibrium in 24 h even at pH 8.0 [30,31]. The effects of disulfides do not result from their transformation to corresponding thiols at the expense of the complex-bound dihydrolipoate either: approximately 10^{-8}–10^{-7} M of the complex-bound lipoate is unable to greatly reduce 10^{-4}–10^{-3} M disulfides during the assay time.

Acknowledgements

This work was partially supported by the Alexander von Humboldt Foundation (Germany). I thank Dr. Hans Bisswanger (Tübingen, Germany) for pyruvate dehydrogenase from *E. coli* and Dr. Aart DeKok (Wageningen, Netherlands) for the 2-oxoglutarate dehydrogenase complex from *A. vinelandii*.

References

[1] A. Berg, A. DeKok, Biol. Chem. 378 (1997) 617–634.
[2] R.N. Perham, in: M.S. Patel, T.E. Roche, R.A. Harris (Eds.), Alpha-Keto Acid Dehydrogenase Complexes, Birkhaeuser Verlag, Basel, Switzerland, 1996, pp. 1–15.
[3] V. Bunik, H. Follmann, H. Bisswanger, Biol. Chem. 378 (1997) 1125–1130.
[4] V. Bunik, G. Raddatz, S. Lemaire, Y. Meyer, J.-P. Jacquot, H. Bisswanger, Prot. Sci. 8 (1999) 65–74.
[5] H. Follmann, I. Häberlein, Biofactors 6 (1995) 147–156.
[6] A. Holmgren, J. Biol. Chem. 264 (1989) 13963–13966.
[7] A. Holmgren, J. Biol. Chem. 254 (1979) 9113–9119.
[8] L.-J. Larsson, A. Holmgren, B. Smedsrod, T. Lindblum, I. Bjork, Biochemistry 27 (1988) 983–991.
[9] O. Schwartz, P. Schürmann, H. Strotmann, J. Biol. Chem. 272 (1997) 16924–16927.
[10] M.K. Geck, F.W. Larimer, F.C. Hartman, J. Biol. Chem. 271 (1996) 24736–24740.
[11] T. Hayashi, Y. Ueno, T. Okamoto, J. Biol. Chem. 268 (1993) 11380–11388.
[12] M.R. Jacquiersarlin, B.S. Polla, Biochem. J. 318 (1996) 187–193.
[13] H. Schenk, M. Vogt, W. Droge, K.J. Schulzeosthoff, Immunology 156 (1996) 765–771.
[14] H. Blum, M. Rollinghoff, A. Gessner, Cytokine 8 (1996) 6–13.
[15] J.R. Gasdaska, M. Berggren, G. Powis, Cell Growth and Differentiation 6 (1995) 1643–1650.
[16] R. Goldman, D.A. Stoyanovsky, B.W. Day, V.E. Kagan, Biochemistry 34 (1995) 4765–4772.
[17] M. Aota, K. Matsuda, N. Isowa, H. Wada, J.J. Yodoi, T. Ban, J. Cardiovasc. Pharmacol. 27 (1996) 727–732.
[18] H. Nakamura, S. Derosa, M. Roederer, M.T. Anderson, J.G. Dubs, J. Yodoi, A. Holmgren, L.A. Herzenberg, Int.. Immunol. 8 (1996) 603–611.
[19] C.J. Stanley, R.N. Perham, Biochem. J. 191 (1980) 147–154.
[20] V.I. Bunik, H. Follmann, FEBS Lett. 336 (1993) 197–200.
[21] V.I. Bunik, O.G. Romash, V.S. Gomazkova, FEBS Lett. 278 (1991) 147–150.
[22] S.D. Varfolomeev, S.V. Zaitsev, in: I.V. Berezin (Ed.), Kinetic Methods in Biochemical Investigations, Moscow State University Publishing Unit, Moscow, Russia, 1982, pp. 105–130.
[23] A.H. Westphal, A. Fabisz-Kijowska, H. Kester, P.P. Obels, A. DeKok, Eur. J. Biochem. 234 (1995) 861–870.
[24] A. DeKok, W.J.H. van Berkel, in: M.S. Patel, T.E. Roche, R.A. Harris (Eds.), Alpha-Keto Acid Dehydrogenase Complexes, Birkhaeuser Verlag, Basel, Switzerland, 1996, pp. 53–70.
[25] L. Sahlman, C.H. Williams Jr., J. Biol. Chem. 264 (1989) 8039–8045.
[26] K.D. Wilkinson, C.H. Williams Jr., J. Biol. Chem. 256 (1981) 2307–2314.
[27] A. Mattevi, G. Obmolova, J.R. Sokatch, C. Betzel, W.G. Hol, Proteins 13 (1992) 336–351.
[28] G. Raddatz, H.J. Bisswanger, Biotechnology 58 (1997) 89–100.
[29] H.R. Schirmer, G.E. Schulz, in: D. Dolphin, R. Poulson, O. Avramovic (Eds.), Pyridine Nucleotides Coenzymes: Chemical, Biochemical and Medical Aspects, Vol. 2B, Wiley, 1987, pp. 333–379.
[30] H.F. Gilbert, J. Biol. Chem. 257 (1982) 12086–12091.
[31] H.F. Gilbert, Meth. Enzymol. 107 (1984) 330–351.
[32] T. Konishi, G. Handelman, S. Matsugo, V.V. Mathur, H.J. Trischler, L. Packer, Biochem. Mol. Biol. Int. 38 (1996) 1155–1161.
[33] J.L. Snoep, M.R. de Graeff, A.H. Westphal, A. De Kok, M.J. Teixeira de Mattos, O.M. Neijssel, FEMS Lett. 114 (1993) 279–284.