AN NADH DEPENDENT CLEAVAGE OF DTNB BY THE α-KETOGLUTARATE DEHYDROGENASE COMPLEX[1]

J. D. Erfle and F. Sauer

Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa 3, Ontario, Canada

Received July 10, 1968

In the titration of sulfhydryl groups in the α-ketoglutarate dehydrogenase complex by the DTNB[2] method of Ellman (1959) it was of interest to determine what contribution to the total sulfhydryl groups was made by reduced, enzyme bound lipoic acid. The mechanism of the reaction predicted that NADH would reduce all

$$\text{RCOCOOH} + \text{TPP-E}_1 \rightarrow [\text{RCH(OH)-TPP}]\text{-E}_1 + \text{CO}_2 \quad (1)$$

$$[\text{RCH(OH)-TPP}]\text{-E}_1 + \text{LipS}_2\text{-E}_2 \rightarrow [\text{RCO-S-LipSH}]\text{-E}_2 + \text{TPP-E}_1 \quad (2)$$

$$[\text{RCO-S-LipSH}]\text{-E}_2 + \text{CoASH} \rightleftarrows [\text{Lip(SH)}_2]\text{-E}_2 + \text{R-CO-S-CoA} \quad (3)$$

$$[\text{Lip(SH)}_2]\text{-E}_2 + \text{E}_3\text{-FAD} \rightleftarrows \text{LipS}_2\text{-E}_2 + \text{reduced E}_3\text{FAD} \quad (4)$$

$$\text{Reduced E}_3\text{-FAD} + \text{NAD}^+ \rightleftarrows \text{E}_3\text{FAD} + \text{NADH} + \text{H}^+ \quad (5)$$

$$\text{RCOCOOH} + \text{NAD}^+ + \text{CoASH} \rightarrow \text{RCO-S-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (6)$$

$$\text{R} = \text{HOOC(CH}_2)_2$$

the lipoic sulfhydryl groups by reversal of reactions 4 and 5 (Sanadi et al. 1952) and these should then be titratable. The reaction of DTNB with sulfhydryl groups in the complex in the presence of NADH proved to be catalytic rather than stoichio metric. The nature of this reaction has been studied and evidence is presented to

---

[1] Contribution No. 317 from the Animal Research Institute.

[2] Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); TNB, 5-thio, 2-nitrobenzoic acid; TPP, thiamine pyrophosphate; LipS_2 and Lip(SH)_2, oxidized and reduced lipoate (or lipoamide); FAD, flavin adenine dinucleotide. E_1 is α-ketoglutarate decarboxylase; E_2 is dihydrolipoyl transuccinylase and E_3 is lipoamide dehydrogenase (E.C. 1.6.4.3).

demonstrate the reaction of DTNB with the lipoic acid sulfhydryl which undergoes succinylation during catalysis of the overall reaction. The dithiol thus formed is reduced by lipoamide dehydrogenase and NADH in an analogous fashion to that of the internal dithiol of lipoic acid (reverse of reaction 4). The significance of this reaction is discussed.

Materials and Methods

The α-ketoglutarate dehydrogenase complex was isolated from bovine heart by the method of Hirashima et al. (1967). The only modification was to replace the cellulose-calcium phosphate gel chromatography with chromatography on a 2.5 x 40 cm column of Sephadex G-200 and elution with 0.05 M potassium phosphate (pH 7.0) containing 0.5 mM EDTA. Traces of pyruvate dehydrogenase were removed by sucrose density centrifugation as described by Ishikawa et al. (1966). The enzyme preparations used in this study had specific activities from 2 to 4 units per mg in the overall reaction (reaction 6).

DTNB was purchased from Aldrich Chemical Co., NADH and iodoacetamide from Sigma Chem. Co. and lipoamide dehydrogenase from Boehringer Mannheim Corp. N-ethyl maleimide was a product of Nutritional Biochemical Corporation.

The method of assay was to follow the increase in absorbancy at 412 mμ in a 1 cm cuvette. Incubations contained potassium phosphate, (pH 7.0), 50 μmoles; NADH, 0.15 μmoles; DTNB, 1 μmole; lipoamide dehydrogenase, 5 μg; and purified α-ketoglutarate dehydrogenase. The details of preincubation and the order of additions are given in the figure and tables.

Results and Discussion

The NADH dependence of the catalytic cleavage of DTNB by the α-ketoglutarate dehydrogenase complex is illustrated in Figure 1 (curve 1). This reaction has pH optima at 7.6 and 9.2. The Km for DTNB was 1 mM at both pH values. The stoichiometry of the reaction was 1 μmole of NADH oxidized per mole of DTNB reduced.

Lipoamide dehydrogenase alone was unable to catalyze this reaction (Figure 1,

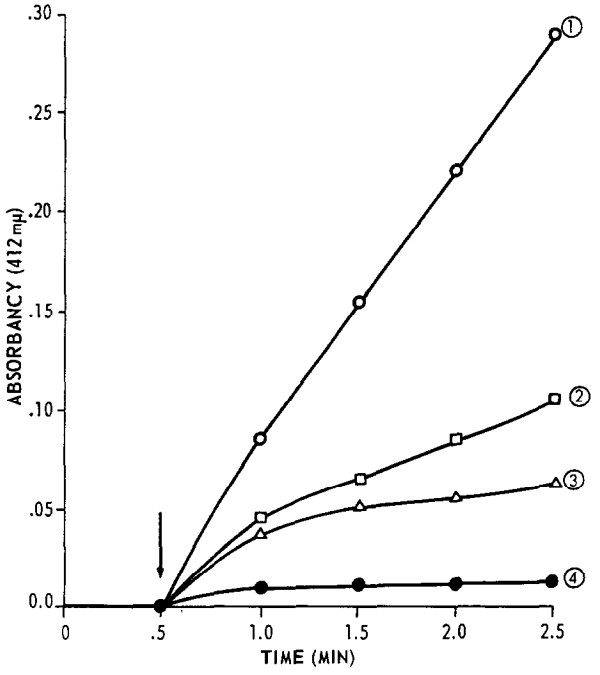


Figure 1. The enzymatic cleavage of DTNB by the α-ketoglutarate dehydrogenase complex. The assay system is described in the Methods. Curve 1 - α-ketoglutarate dehydrogenase, 53 μg, (Sp. Act. 4 units/mg); curve 2 - same as 1 but preincubated 2 min. with NADH and succinyl-CoA (0.098 μmoles/ml); curve 3 - same as 1 but preincubated 2 min. with α-ketoglutarate (0.1 μmole/ml); curve 4 - lipoamide dehydrogenase (25 μg). At the point indicated by the arrow the reaction was started by the addition of NADH (curves 1 and 4) or DTNB (curves 2 and 3).

Table I. Properties of the α-ketoglutarate dehydrogenase catalyzed cleavage of DTNB.

Enzyme additions*	Other additions	% Activity
α-KGDH (native)	none	100
Lip.DH	none	0
Lip.DH	lipoamide (0.01 μmole)	780
α-KGDH (denatured)** + Lip.DH	none	78
α-KGDH (denatured) + Lip.DH	Succinyl-CoA	78
α-KGDH (denatured) + Lip.DH	α-ketoglutarate	100

* The complete system is described in Methods. The reaction was run at pH 7.0, and initiated with DTNB. α-KGDH is α-ketoglutarate dehydrogenase complex. Lip.DH is lipoamide dehydrogenase.

** α-KGDH was denatured by a 10 min. incubation in 2.5 N KOH of 30°. The preparation was neutralized to pH 7.0 and buffered before assaying.

curve 4), unless catalytic quantities of lipoamide were added (Table I). Thus the requirement for the remainder of the α-ketoglutarate dehydrogenase complex probably resides with its protein bound lipoic acid residues (i.e. in the dihydrolipoyl transuccinylase). That such is the case is demonstrated in Table I where lipoamide dehydrogenase promotes DTNB cleavage in the presence of denatured α-ketoglutarate dehydrogenase complex.

The participation of the lipoic acid moieties of dihydrolipoyl transuccinylase in DTNB cleavage is further demonstrated (Figure 1, curves 2 and 3) by the inhibition which results from preincubation of the enzyme complex with α-ketoglutarate or succinyl-CoA plus NADH. This results in enzymatic succinylation of the lipoic acid in the enzyme by reactions 1 and 2 in the case of α-ketoglutarate and by reversal of reactions 3-5 in the case of succinyl-CoA and NADH. Neither α-ketoglutarate nor succinyl-CoA was inhibitory when the complex was denatured (Table I). Additional evidence for the role of lipoic acid in the cleavage of DTNB is provided by

Table II. Effect of NADH on NEM and iodoacetamide inhibition of DTNB cleavage by the α-ketoglutarate dehydrogenase complex

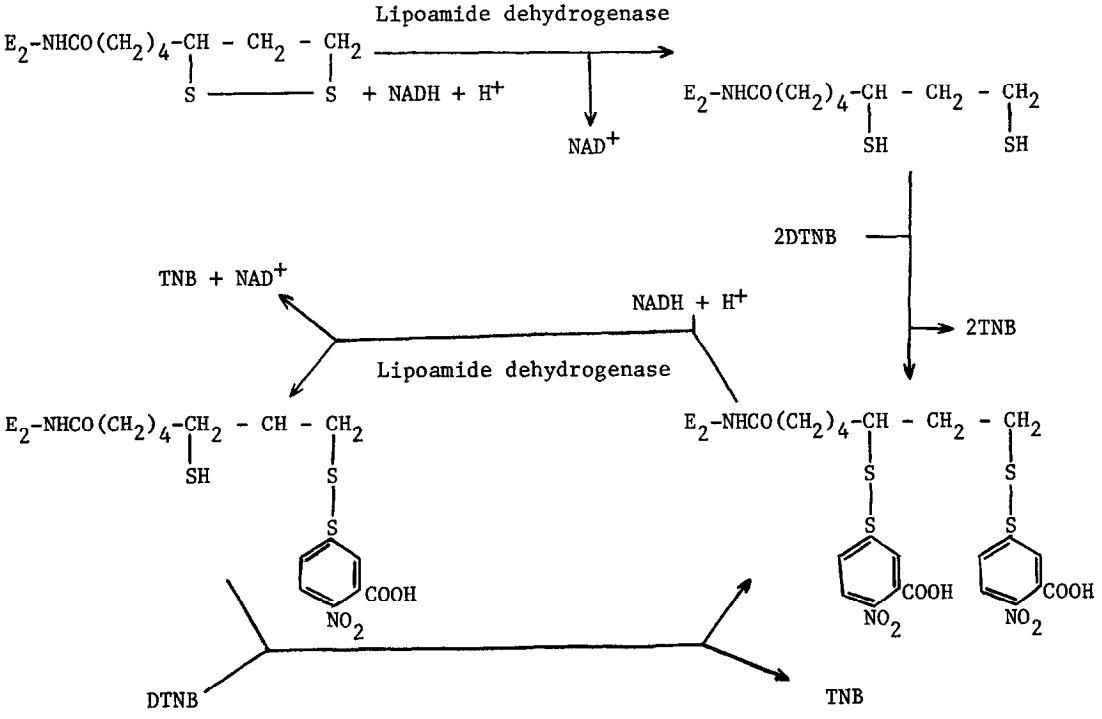
Preincubation conditions:*			
NADH	NEM	Iodoacetamide	Enzyme activity** (%)
-	-	-	100
-	+	-	95
+	+	-	0
-	-	+	78
+	-	+	0

* Preincubations contained in 1 ml: Tris-Cl (pH 7.0), 50 μmoles; EDTA, 0.2 μmoles; NADH, 1.2 μmoles; NEM, 1 μmole; iodoacetamide, 2 μmoles; αKGDH, 3.6 mg. A 10 min. preincubation of enzyme and NADH was followed by a 15 min. incubation after the addition of sulfhydryl reagents. After the incubation a 0.25 ml aliquot of the last four incubations was mixed with a stoichiometric amount of cysteine to titrate sulfhydryl reagents.

** The enzyme preparations were assayed as described in Methods. Lipoamide dehydrogenase (5 μg) was added to each assay. The reactions were started by the addition of NADH.

the inhibition studies with the sulfhydryl reagents N-ethylmaleimide and iodoacetamide (Table II). Inhibition by both compounds is dependent on NADH and hence on the reversal of reactions 4 and 5. These results also show that the native α-ketoglutarate dehydrogenase complex contains only oxidized lipoic acid residues.

The proposed mechanism of DTNB cleavage is shown as one in which lipoamide dehydrogenase cleaves the disulfide bond between lipoic acid and thionitrobenzoate. Since the sulfhydryl at carbon 6 of lipoic acid is the one reported to undergo



succinylation (Gunsalus et al., 1958) it is depicted as the one involved in the reaction. This is consistent with inhibition caused by conditions favouring succinylation.

Conclusions

The results presented demonstrate the NADH dependent cleavage of the lipoic acid-TNB disulfide by lipoamide dehydrogenase. Inhibition by conditions conducive to succinylation have led to the proposed mechanism. This model reaction has been

used to study the involvement of lipoic acid moieties in the inhibition of the overall reaction by long chain acyl-CoA esters and may have application in studies of other types of inhibition as well. The reaction may in addition provide a useful tool for demonstrating the catalytic involvement of protein bound lipoic acid in other enzymes.

This enzyme-catalyzed cleavage of a dithiol formed from reaction with DTNB is to the best of our knowledge a hitherto unreported reaction. The possibility that this reaction is of a more general or non-specific nature should serve as a caution to investigators who attempt the titration of enzyme sulfhydryl groups in the presence of substrate.

References

Ellman, G. L., (1959), Archives Biochem. Biophys., 82, 70.
Gunsalus, I. C., and Smith, R. A., (1958), in Proceedings of the International Symposium on Enzyme Chemistry, p. 77. Maruzen, Tokyo.
Hirashima, M., Hayakawa, T., and Koike, M., (1967), J. Biol. Chem. 243, 902.
Ishikawa, E., Oliver, R. M., and Reed, L. J., (1966), Proc. Natl. Acad. Sci. (U.S.) 56, 534.
Sanadi, D. R., Littlefield, J. W., and Bock, R. M., (1952), J. Biol. Chem. 197, 851.