INACTIVATION OF THE PYRUVATE DEHYDROGENASE COMPONENT FROM PIGEON BREAST MUSCLE PYRUVATE DEHYDROGENASE COMPLEX BY α-KETOBUTYRIC ACID

Gerhard HÜBNER and Alfred SCHELLENBERGER
Sektion Biowissenschaften, Wissenschaftsbereich Biochemie (Enzymologische Abteilung), Martin-Luther-Universität, Halle (Saale), GDR

and

Rita BERNHARDT, Ludmila S. KHAILOVA and Sergei E. SEVERIN
Department of Biochemistry, Lomonosov University, Moscow, USSR

Received 5 August 1977
Revised version received 3 October 1977

1. Introduction

The pyruvate decarboxylating component of the pyruvate dehydrogenase complex (PDH, EC 1.2.4.1.) from pigeon breast muscle catalyses the oxidative decarboxylation of pyruvate to acetate and CO_2 in the presence of hydrogen acceptors such as dichlorphenolindophenol (DCPIP; [1]).

The activity of the pyruvate decarboxylating component depends on the cofactors thiamine pyrophosphate (TPP) and magnesium ions, both bound reversibly to the protein component. This is in contrast to pyruvate decarboxylase (EC 4.1.1.1.) where an irreversible fixation of the same cofactors could be established [2].

If α-ketobutyrate is used as substrate of the pyruvate decarboxylating component, inactivation occurs after a short period of high activity. By means of a kinetic analysis of the α-ketobutyrate-induced inhibition reaction the presented paper reveals some new aspects concerning the mechanism of the PDH reaction.

Address: Professor Dr A. Schellenberger, Wissenschaftsbereich Biochemie (Enzymolog. Abtlg.) der Sektion Biowissenschaften, Universität Halle (Saale), DDR-402 Halle (Saale), Domplatz 1, GDR

2. Materials and methods

The pyruvate decarboxylating component was prepared from the pigeon breast muscle complex by KBr-treatment [3] using a modified method [4] of Jagannathan and Scheet [5] (spec. act. 0.29 U/mg).

α-Ketobutyric acid (synthesized according to [6] and pyruvic acid (Sigma) were purified by distillation in vacuo. α-[3-$^2H_2$] Ketobutyric acid was prepared by deuteration of the ethyl ester with ^2H_2O followed by hydrolysis of the ester using $^2HCl/^2H_2O$ (1 N). The completeness of deuteration was proved by NMR spectroscopy.

2-α-hydroxypropyl-TPP was prepared by incubation of TPP with butyric aldehyde, at pH 8.9, according to Holzer [7] and purified by chromatography via Dowex 2 × 8 (formiate form) taking a formic acid gradient for elution [8].

TPP and DCPIP were commercial preparations (Sigma), all other substances were of p.a. purity.

The kinetic experiments were performed using DCPIP as hydrogen acceptor. The decrease of extinction at 600 nm was taken for estimation of activity. The experiments were carried out in 0.05 M potassium phosphate buffer, pH 7.0, at 25°C using a Hitachi (M 356) spectrophotometer. Incubation mixtures

(3 ml) contained 80 μg pyruvate decarboxylating component and the substrate; they were 3.3×10^{-5} M in TPP, 3.3×10^{-4} M in magnesium chloride and 10^{-4} M in DCPIP.

3. Results

Treatment of the PDH with α-ketobutyrate as substrate causes in the beginning of the measurement a quick reaction which is followed by a rather fast decrease of the catalytic activity of the enzyme. The same behaviour is observed with the pyruvate decarboxylating component. The residual activity reaches a constant value (fig.1, curve A) which depends on the α-ketobutyrate concentration. Inactivation is a first order reaction (fig.1, curve B) and requires not only the presence of the cofactors TPP and Mg^{2+} but also the hydrogen acceptor DCPIP (table 1).

A smaller decrease of the activity of the pyruvate decarboxylating component after the starting phase of the enzyme reaction is observed with pyruvate as substrate (fig.1, curve C).

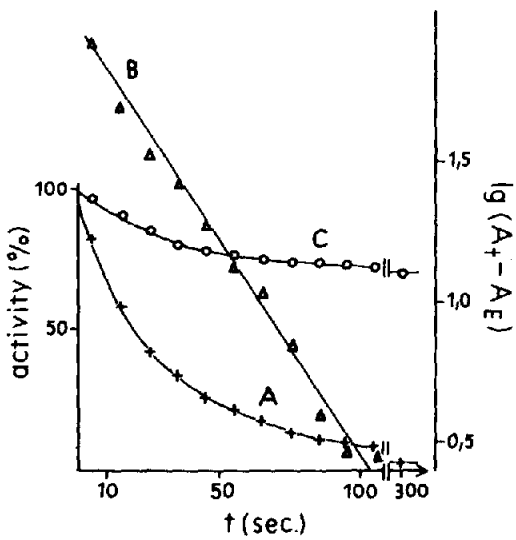

Fig.1. Rate/activity curves of the α-ketobutyrate degradation (curve A) and pyruvate degradation (curve C) by the pyruvate decarboxylating component of PDH. Curve B: log activity/time curve of the α-ketobutyrate degradation. A_t, Activity to time t; A_E, Constant end activity. Concentrations: α-ketobutyrate, 3.3×10^{-4} M; pyruvate, 3.3×10^{-4} M.

Table 1
Inhibitory effect of α-ketobutyrate on the equilibrium activity of the pyruvate decarboxylating component of PDH

TPP	Mg^{2+}	DCPIP	α-Keto-butyrate	Activity (%)
+	+	+	–	100
–	–	-	+	97
+	+	–	+	94
-	–	+	+	95
+	+	+	+	7

After 4 min pre-incubation with α-ketobutyrate (conc. of α-ketobutyrate = 6.7×10^{-5} M) and completing the system with the missing components and pyruvate the activities were measured and related to the activity without α-ketobutyrate (100%)

The dependence of the inactivation rate constant on the α-ketobutyrate concentration is shown in fig.2 (curve A). The same figure shows a plot of the initial enzyme activity against the α-ketobutyrate concentration (curve B). Simultaneous addition of pyruvate plus α-ketobutyrate causes a decrease of the inactivation rate (fig.3) with increasing concentration of α-ketobutyrate, showing a competition of the two substrates in the inactivation mechanism.

Incubation of the α-ketobutyrate-inactivated pyruvate decarboxylating component with pyruvate causes a slow and complete reactivation of the

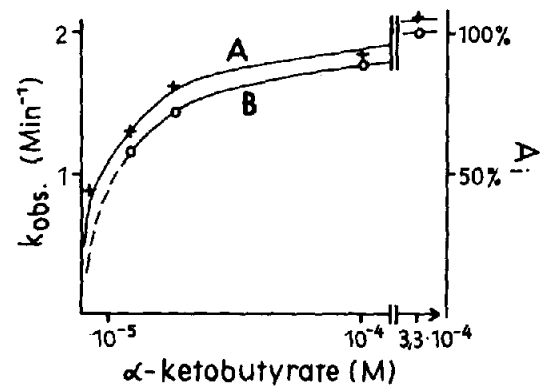

Fig.2. Influence of the α-ketobutyrate concentration on the rate constant of the inactivation reaction k_{obs} (curve A) and dependence of the initial rate A_i on the α-ketobutyrate concentration (curve B).

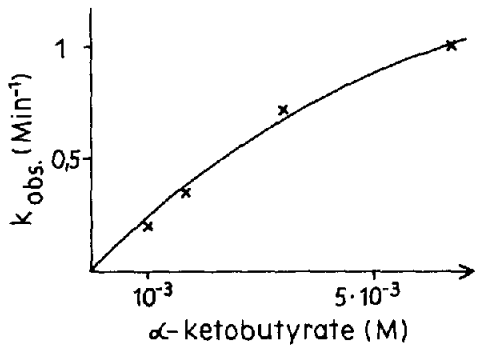

Fig.3. Influence of the α-ketobutyrate concentration on the rate constant of the inactivation reaction k_{obs} in the presence of 1.67×10^{-3} M pyruvate.

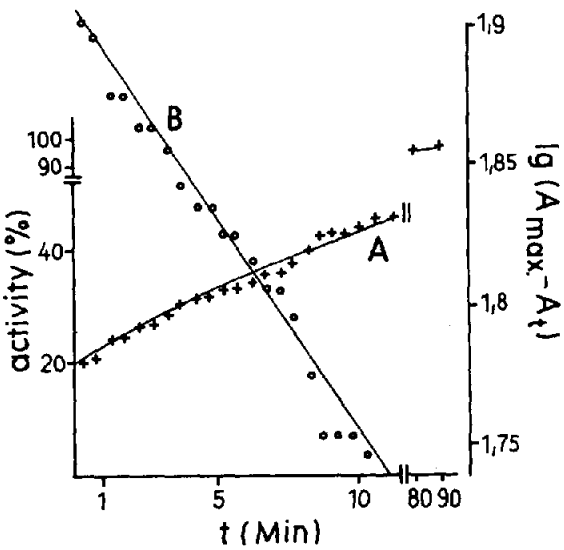

Fig.4. Reactivation of the α-ketobutyrate inactivated pyruvate decarboxylating component by pyruvate (curve A). Incubation mixture for inactivation: 3.3×10^{-5} M TPP, 3.3×10^{-3} M Mg^{2+}, 5×10^{-4} M DCPIP, 1.67×10^{-3} M α-ketobutyrate. Volume: 100 μl with 80 μg pyruvate decarboxylating component/assay. After 1 min incubation the assay was diluted to 3 ml and after another 30 s TPP, Mg^{2+} and DCPIP were added (at concentrations mentioned under methods) and pyruvate was added to reach 1.67×10^{-3} M in the assay mixture. Curve B: log ($A_{max}-A_t$)/time curve of the reactivation reaction. A_{max}: Activity reached after complete activation. A_t: Activity to time t.

enzyme. The rate constant of this first order reactivation reaction is 3.5×10^{-2} min^{-1} (fig.4).

As the decarboxylation of α-ketobutyrate (i.e., the first step of the enzyme reaction with the pyruvate decarboxylating component) proceeds very probably via 2-α-hydroxypropyl-TPP, another series of experiments was made using this intermediate as substrate of the enzyme reaction. The same inactivation of the pyruvate decarboxylating component occurs in the presence of DCPIP under saturation conditions with 2-α-hydroxypropyl-TPP (3.3×10^{-3} M) showing a first order rate constant of k_{obs} 1.95 min^{-1}. This is about the same value which is found using α-ketobutyrate as substrate under saturation conditions (k_{obs} 2.1 min^{-1}, fig.2).

Inactivation of the pyruvate decarboxylating component using [3-$^2H_2$]-α-ketobutyrate as substrate results in a reduced rate of the enzyme reaction in comparison to the undeuterated substrate. The isotopic effect on the rate constants of inactivation k_H/k_D is 1.7 and is independent of the α-ketobutyrate concentration.

4. Discussion

Inactivation of the pyruvate decarboxylating component of PDH by α-ketobutyrate is a reversible reaction (fig.4) and leads in accordance with the constant residual activity to an equilibrium between active and inactive species of the enzyme. In this equilibrium only 3% of the total enzyme concentration are present in the active state (fig.1) which is in good agreement with the ratio of the observed rate constants for the reactivation (k_{obs} $3.5 \cdot 10^{-2}$ min^{-1}) and inactivation (k_{obs} 2.1 min^{-1}) reactions. As the inactivation reaction requires the presence of the cofactors (TPP and Mg^{2+}) and DCPIP (table 1) the reaction which produces the inactive species of the enzyme should start from the level of the acyl-(propionyl) enzyme.

The rate constant of the reactivation reaction with pyruvate (k_{obs} 3.5×10^{-2} min^{-1}) is considerably smaller than the rate constant of the cofactor dissociation (k_{diss} 1.9 min^{-1} [9]) showing either the acyl residue to be no longer fixed to the TPP molecule in the inactive acyl-enzyme species or the 2-propionyl-TPP molecule to be fixed more stable to the protein

than the TPP-molecule. The reactivation reaction is also observed if α-ketobutyrate is separated from the reaction mixture by Sephadex G-25 filtration showing that the reactivation reaction is not influenced by the pyruvate molecule.

In the case of the propionyl enzyme derivate the equilibrium between active and inactive enzyme species is mainly shifted to the inactive species (fig.1, curve A). Contrary to this the acetyl-enzyme derivative resulting from the pyruvate reaction contains only 30% of the inactive species in the equilibrium (fig.1, curve C). The initial rate of the α-ketobutyrate turnover (estimated from the initial slopes of the turnover plots) as well as the rate of inactivation are proportional to the steady state concentration of the acyl enzyme complex. Therefore both parameters show saturation curves with identical concentrations of half-saturation (fig.2).

The experiments with α-ketobutyrate as substrate show that in the oxidative decarboxylation of α-keto acids by means of PDH a side reaction is possible on the level of the acyl enzyme complex producing an inactive species which is incapable of splitting into reaction product and the free enzyme. The isotopic effect of 1.7 (primary isotopic effect!) shows that the splitting off of a proton which is α-positioned to the carbonyl group, i.e., the enolization of the propionyl (acyl) enzyme species determined the rate of the inactivation reaction.

References

[1] Das, M. L., Koike, M. and Reed, L. J. (1961) Proc. Natl. Acad. Sci. 47, 753.
[2] Schellenberger, A. (1967) Angew. Chem. 79, 1050.
[3] Khailova, L. S., Glemsha, A. A. and Severin, S. E. (1970) Biokhimia 35, 536.
[4] Fligina, M. M. (1973) Dissertation, Moscow University.
[5] Jagannathan, V. and Scheet, R. C. (1952) J. Biol. Chem. 196, 551.
[6] Fischer, G. (1970) Dissertation Halle/S. University.
[7] Holzer, H., Goedde, H. W. and Ullrich, J. (1961) Biochem. Biophys. Res. Commun. 5, 447.
[8] Schellenberger, A. and Hübner, G. (1965), Hoppe-Seyler's Z. Physiol. Chem. 343, 189.
[9] Paper in preparation.