EFFECT OF PYRUVATE AND ITS ANALOGS ON THE THIAMINE PYROPHOSPHATE BINDING IN THE ACTIVE CENTER OF MUSCLE PYRUVATE DEHYDROGENASE

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1. Introduction

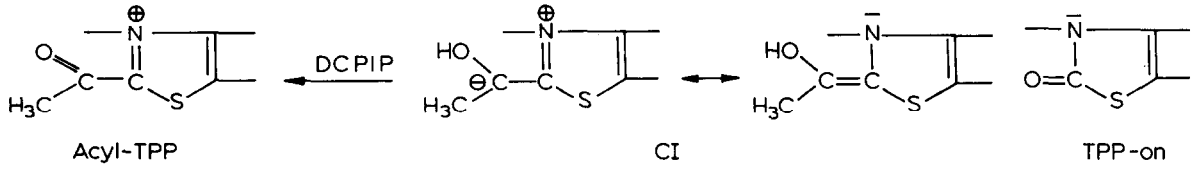
The pyruvate decarboxylating component (PDC) of the pyruvate dehydrogenase complex from pigeon breast muscle (EC 1.2.4.1) catalyzes the oxidative decarboxylation of pyruvate in the presence of thiamine pyrophosphate (TPP), Mg^{2+} and non-physiological oxidants, such as 2,6-dichlorophenol-indophenol (DCPIP), with the formation of CO_2, acetate and the reduced dye [1,2]. The 'α-carbanion'-intermediate (CI) formed in the enzyme reaction after the decarboxylation of the TPP-bound substrate is oxidized by the dye [3]. The protonated intermediate (2-(α-hydroxyethyl)-TPP, HETPP) shows no significant reactivity with DCPIP.

This paper confirms that in the course of the inactivation reaction, an inactive complex between the protein and a TPP-derivative is produced, which is formed in the oxidation reaction with DCPIP. The carbanion intermediate with its reduced positive charge on the thiazolium ring is bound more tightly to the active center than the coenzyme itself.

2. Materials and methods

α-Ketobutyric acid synthesized according to [6] and pyruvic acid (Sigma) were purified by distillation in vacuo. ^{32}P-Labelled TPP was prepared by phosphorylation of thiamine using $H_3{}^{32}PO_4$ according to [7]. ^{32}P-Labelled HPTPP was obtained from [^{32}P]TPP by incubation with butyric aldehyde (pH 8.9) according to [8] and purified by chromatography via Dowex 2 X 8 (formiate form), using a formic acid gradient for elution [9]. TPP and DCPIP of commercial purity grade were purchased from Sigma, all other reagents were of p.a. purity.

The pyruvate decarboxylating component was obtained in the form of the apoenzyme from a highly purified preparation of pigeon breast muscle pyruvate dehydrogenase complex by treatment with KBr [4,5]



If α-ketobutyrate is used as substrate of the decarboxylating component, a rapid inactivation of the enzyme occurs after a short period of high activity [4]. On principle this effect can be observed also with pyruvate but in a much smaller degree than in the case of the substrate analog (fig.1). This inactivation reaction proceeds only in the presence of TPP, Mg^{2+} and DCPIP, suggesting the formation of a relatively stable non-productive complex between the protein component and the oxidized species of the 2-(α-hydroxypropyl)-TPP-carbanion (HPTPP(−)) [5].

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* Dedicated to Professor A. E. Braunstein on the occasion of his 80th birthday

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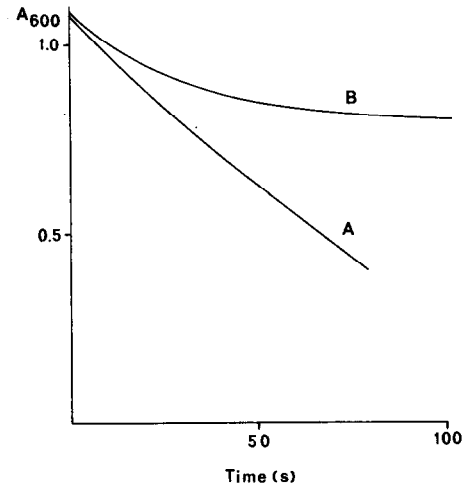

Fig.1. Progress curves of the PDC-catalysed oxidative decarboxylation of pyruvate (curve A) and α-ketobutyrate (curve B) by DCPIP. Conditions: PDC, 80 μg/ml 0.05 M potassium phosphate buffer (pH 7.0); 3.3×10^{-5} M TPP; 3.3×10^{-4} M $MgCl_2$; 3.3×10^{-4} M pyruvate; 3.3×10^{-4} M α-ketobutyrate; 5.5×10^{-5} M DCPIP. Pathlength 1 cm; temp. 25°C.

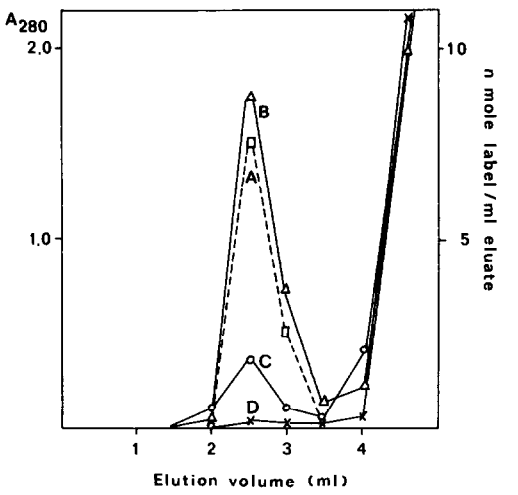

Fig.2. Elution profiles of the PDC after incubation with the labelled cofactors (D), with cofactors and pyruvate (C), and with cofactors, α-ketobutyrate and DCPIP (B). Protein absorption at 280 nm, pathlength 1 cm (A).

with a specific activity of the component after recombination of 0.35 U/mg.

The apoenzyme was incubated with substrates and cofactors under the specific conditions listed in the table and the protein fractions were separated from low M_r reagents on a column containing Sephadex G-75 fine, 22 × 0.5 cm. Fractions of 0.5 ml were collected. Protein absorption and radioactivity were estimated in the eluates.

3. Results

Fig.2 shows the elution profiles, which are obtained under the conditions summarized in the table (exp. 1–7). As described in exp. 1, the holoenzyme formed by incubation of the PDC with the cofactors (TPP and Mg^{2+}) dissociates practically completely after removal of the excess coenzyme by gel filtration. After preincubation of the holoenzyme with pyruvate the amount of [^{32}P]TPP in the protein fraction increases significantly (exp. 2). Another increase of the ^{32}P-content in the protein is observed, if the gel filtration of exp. 2 is performed in the presence of excess pyruvate in the incubation and gel filtration steps (exp. 3).

The same assay in the presence of DCPIP results in a marked decrease of the amount of labelled TPP in the protein fraction even in the presence of excess pyruvate in the incubation and gel filtration steps (exp. 4).

However, a considerable increase of the amount of tightly bound ^{32}P in the protein fraction is observed after incubation of the PDC with α-ketobutyrate

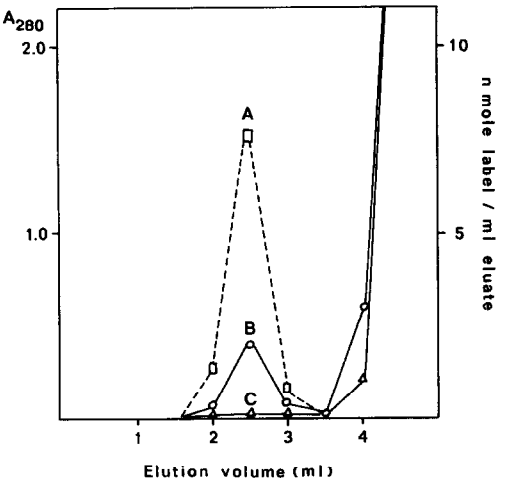

Fig.3. Elution profiles of the pyruvate decarboxylating component after incubation with cofactors and [1-^{14}C]pyruvate (C) and [2-^{14}C]pyruvate (B). Protein absorption at 280 nm, pathlength 1 cm (A).

Table 1
Amount of radioactive label in the eluted protein fractions after gel filtration of the pyruvate decarboxylase component

No. exp.	PDC (M)	$MgCl_2$ (M)	[^{32}P]TPP (M)	Pyruvate (M)	DCPIP (M)	α-Keto-butyrate (M)	[^{32}P]HPTPP (M)	[2-^{14}C]-pyruvate (M)	[1-^{14}C]-pyruvate (M)	Mol label/Mol enzyme (in protein peak)
1	1.2×10^{-5}	3.3×10^{-3}	3.3×10^{-4}							0.01
2	1.2×10^{-5}	3.3×10^{-3}	3.3×10^{-4}	3.0×10^{-2}						0.17
3	1.2×10^{-5}	3.3×10^{-3}	3.3×10^{-4}	3.0×10^{-2}						0.19[a]
4	1.2×10^{-5}	3.3×10^{-3}	3.3×10^{-4}	3.0×10^{-2}	5.0×10^{-4}					0.10[b]
5	0.9×10^{-5}	0.7×10^{-3}	3.6×10^{-4}			5.0×10^{-3}				0.86
6	1.2×10^{-5}	3.3×10^{-3}	3.3×10^{-4}		5.0×10^{-4}	5.0×10^{-3}				0.66
7	1.2×10^{-5}	3.3×10^{-3}			5.0×10^{-4}		3.3×10^{-4}			0.86
8	1.5×10^{-5}	5.0×10^{-3}	1.7×10^{-3} [c]					1.0×10^{-3}		0.20
9	1.5×10^{-5}	5.0×10^{-3}	1.7×10^{-3} [c]						1.0×10^{-3}	0.005

[a] Buffer containing 10^{-3} M pyruvate
[b] Buffer containing 10^{-3} M pyruvate and 5.0×10^{-4} M DCPIP
[c] With unlabelled TPP

Gel filtration medium: 0.05 M potassium phosphate buffer (pH 7.0)
The enzyme was incubated with the different cofactors, substrates and substrate analogs as in section 2

instead of pyruvate. The amount of ^{32}P in the eluate reaches 0.86 mol/mol enzyme (exp. 5). This ratio reduces only slightly (insignificantly) after addition of DCPIP (exp. 6). This stabilizing effect is also observed, when ^{32}P-labelled HPTPP (the intermediate of the α-ketobutyrate conversion) is used as substrate (exp. 7).

Experiments with labelled pyruvate (instead of the labelled cofactor) led to a confirmation of the results. After addition of [1-^{14}C]pyruvate to the assay mixture no incorporation of radioactivity into the protein is observed, i.e., $^{14}CO_2$ separates quantitatively during the gel filtration step (exp. 9). However, the incubation of the enzyme with [2-^{14}C]pyruvate causes an incorporation of the labelled material into the protein fraction (exp. 8). The amount of radioactivity obtained in the protein peak (fig.3) was comparable to the ^{32}P-content of the protein under similar experimental conditions.

4. Discussion

The pyruvate decarboxylating component of the pyruvate dehydrogenase complex catalyzes the initial step of the complex reaction, i.e., the decarboxylation of pyruvate [10] with the formation of the 2-(α-hydroxyethyl)-TPP carbanion (CI) [11]. In the absence of DCPIP the reaction of the decarboxylating compo nent is blocked on the level of the CI-state, which (as shown by the resonance structure) is characterized mainly by the reduced positive charge on the thiazolium ring.

From the exp. 2, 3 and 4 (table 1) it must be concluded that the high TPP-binding capacity of the PDC, which is observed only in the presence of the substrate alone (DCPIP reduces the ^{32}P-binding capacity!) refers to the CI-state. This result is strongly supported by the fact that the protein binds comparable amounts of ^{32}P- or ^{14}C-labelled CI under similiar conditions (exp. 8). However, in [12], the protonated form of the CI (HETPP), which carries the full positive charge on the thiazolium ring, eliminates easily from the protein component.

These findings are in full agreement with results in [13], where the thiazolon derivative of TPP (TPP-on), which carries (like the CI) a reduced (missing) positive charge on the thiazole ring, showed a considerably higher affinity to the active site of the pyruvate dehydrogenase complex of *E. coli* than TPP itself. TPP-on has been regarded therefore as a transition state (TS) analog, showing that the reduced positive charge on the thiazolium ring is a characteristic of the TS.

In the presence of DCPIP, a degradation of the CI to TPP and acetate via the acylated form of TPP occurs. This returning of the CI to the TPP-form allows the dissociation of the coenzyme from the protein, which is observed in exp. 4.

Besides this 'normal' enzyme reaction, a side reaction of the CI-oxidation with DCPIP, which produces a stable (irreversibly bound) protein complex with a CI-oxidation product, is observed with pyruvate as substrate (fig.1). The amount of this 'abnormal' TPP-derivative binding reaction increases considerably if α-ketobutyrate (exp. 5) or HPTPP (exp. 7) is taken as substrate.

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