Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 517-521

# CHARACTERIZATION OF THE INHIBITION OF *ESCHERICHIA COLI* PYRUVATE DEHYDROGENASE COMPLEX BY PYRUVATE*

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Received March 6, 1991

The *E. coli* pyruvate dehydrogenase complex was inhibited by pyruvate in absence of its cofactor, $NAD^+$. The inhibition was found to increase with pH and phosphate concentration of the buffer and decrease with its ionic strength. The inhibition profile was different with MOPS buffer. No radioactivity was found in the enzyme, when the latter was incubated with 2-$^{14}$C-pyruvate. The results suggest that covalent adduct formation is not necessary for the observed inhibition.


The pyruvate dehydrogenase (PDH) multienzyme complex of *Escherichia coli* catalyzes the decarboxylation and dehydrogenation of pyruvate through a sequence of complex reactions. The overall reaction catalyzed by this enzyme complex is the following:

$$\text{Pyruvate} + \text{CoASH} + NAD^+ \text{---> } CO_2 + \text{Acetyl CoA} + \text{NADH}$$

Three enzymes comprising the complex take part in the sequence of reactions leading to the final products. These enzymes are: pyruvate dehydrogenase ($E_1$), which requires thiamin pyrophosphate (TPP) for its activity; dihydrolipoyl transacetylase ($E_2$), which contains covalently bound lipoic acid; and dihydrolipoyl dehydrogenase ($E_3$), a flavoprotein containing non-covalently bound FAD.

Khailova *et al.* (1) reported a substrate-dependent inactivation of pigeon breast muscle PDH complex in the absence of electron acceptors. The inactivation was shown to result in modification of two sulfhydryl groups per mole of the enzyme. Flournoy and Frey (2) have shown a rapid inactivation of PDH complex and its $E_1$ component in the presence of fluoropyruvate, an unusual substrate for the enzyme. Recently, Williams *et al*. (3) reported a similar inactivation of pyruvate:ferredoxin oxidoreductase from *Trichomonas vaginalis* by pyruvate and its analogs.

The present work deals with the inactivation of PDH complex isolated from *E. coli*, and its subsequent characterization. The inactivation profiles of the enzyme complex were studied in different buffer conditions and their rate constants were determined. The mechanism of inactivation was further investigated using radiolabeled pyruvate.

*The work was performed at the Institute for Enzyme Research, Madison, Wisconsin 53705.

Abbreviations: $NAD^+$ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; TPP, thiamin pyrophosphate; acetyl-TPP, 2-acetylthiamin pyrophosphate; FAD, flavin adenine dinucleotide; MOPS, 3-(N-morpholino)-propanesulfonic acid.

0006-291X/91 $1.50

## MATERIALS AND METHODS

The PDH complex was purified by the method described by Reed and Willms (4), as modified by Speckhard and Frey (5). All substrates, cofactors and chemicals were purchased from Sigma. Dihydrolipoamide was prepared by reducing *dl*-lipoamide (Sigma) with sodium borohydride, according to Reed *et al*. (6). The [2-$^{14}$C]pyruvate was purchased from New England Nuclear and was further purified prior to use by anion-exchange chromatography using the resin Bio-Rad AG1-X4 ($Cl^-$), as detailed in Gruys *et al.* (7). The PDH complex was assayed according to Maldonado (8). The $E_2$ component was assayed with the reaction mixture as described by Yang and Frey (9), and the acetylthioester formed was assayed according to Reed and Willms (4). The $E_3$ component was assayed according to Yang and Frey (9). The separation of reaction products was achieved by Sephadex G-25 chromatography with 5 mM potassium phosphate (pH 7.0).

The spectral measurements were performed with a Cary 118 UV-vis spectrophotometer. Radioactivity ($^{14}$C) was measured by using a Packard Tri-Carb Model 4640 scintillation spectrometer set to the $^{14}$C window (93% efficiency). The reaction mixtures for inactivation studies were incubated at 25 °C in a temperature controlled water bath. Inactivation reaction was started by adding pyruvate and aliquots were taken with a Hamilton syringe at appropriate times for assay. Anaerobic inactivation measurements were carried out in a specially constructed glass chamber, sealed with a rubber septum, and deoxygenated by repeated evacuation and purging with argon. Pyruvate was added with a Hamilton syringe through the rubber septum to start the reaction.

## RESULTS AND DISCUSSION

Initially, the conditions which would result in maximal inactivation were determined using varied concentrations of pyruvate and TPP. It was found that about 80% inactivation in 35 min could be achieved with 0.2 mM pyruvate and 0.1 mM TPP at 25 °C with no NAD present. The extent of inactivation decreased with higher amount of pyruvate, probably due to the carboligase reactions leading to the formation of acetolactate. In all these experiments the control (enzyme alone) did not show any drop in activity over the time span. The $E_2$ or $E_3$ component of the enzyme complex did not show any drop in activity under these conditions. The presence of TPP was required for inactivation. When TPP was left out, slight inactivation was observed, which can be attributed to the endogeneous TPP bound to the enzyme complex. The inactivation was less under anaerobic conditions. No significant difference in inactivation rates was observed in pure oxygen versus air.

### Effect of pH

The inactivation profiles were studied at different pHs using phosphate buffers, as shown in Fig. 1A. The data were collected in all these experiments using 50 mM phosphate buffer, the ionic strengths of the buffers being kept at a nominal value of 0.1. Fig. 1B shows the variation of rate constant of inactivation with pH. The profile does not appear to be linear and shows an enhancement in the rate of inactivation above pH 7.0, suggesting the generation of highly reactive phosphate dianions in that pH range.

### Effect of ionic strength and phosphate concentrations

The rates of inactivation were determined at different ionic strengths and phosphate concentrations (Figs. 2 and 3). Although the rate constant decreased with increase in ionic strength, it rose linearly with increase in phosphate concentration over the range studied. In the latter measurements, the ionic strengths of the buffers were kept constant at 0.3 by the addition of

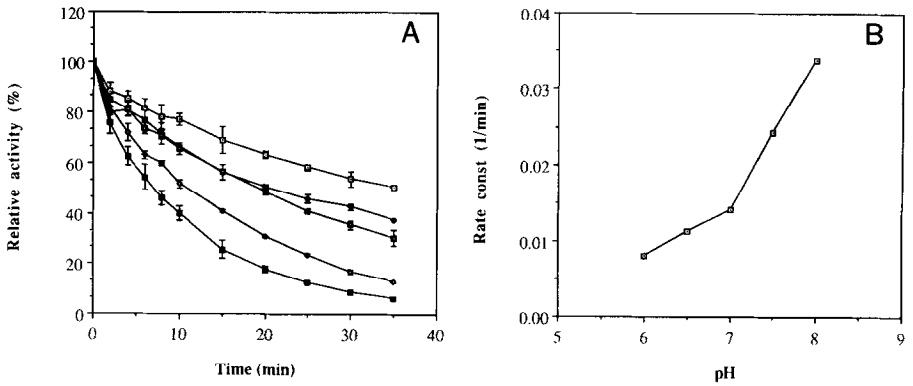


Fig. 1A. The inactivation profile with time at different pHs. The reaction mixture contained 0.2 mM pyruvate, 0.1 mM TPP, 1 mM $MgSO_4$, 0.071 mg/ml PDH complex in a total volume of 0.2 ml in 50 mM potassium phosphate buffers at indicated pHs. The ionic strengths of the buffers were kept constant at 0.1 by the addition of KCl. The solution containing everything except pyruvate was incubated in the bath at 25 °C for 5 min for temperature equilibration before zero time point was assayed. Then pyruvate was added and 10 µl aliquots were withdrawn at definite intervals of time and assayed for PDH complex activity. The symbols are as follows: pH 6.0 (⊡), pH 6.5 (♦), pH 7.0 (■), pH 7.5 (◈), pH 8.0 (■). Data represent mean of triplicate measurements with bars showing the standard deviations. The activity was normalized with respect to the zero point value, which was taken as 100%.

Fig. 1B. Rate constant as a function of pH. Rate constants were determined from semilog plots of Fig. 1.

KCl. A striking buffer effect was observed when the inactivation in phosphate buffer was compared with that in MOPS containing 0.021 mM phosphate (Fig.4). The latter was added to MOPS in an amount which is three times as high as comes with the enzyme during its purification. With phosphate a steady linear fall in activity was discerned even after one hour of incubation, while with MOPS (plus phosphate), an initial rapid drop was followed by a slow levelling out. When a second aliquot of pyruvate was added, another burst of drop in activity was observed. So, the plateau reached with MOPS (plus phosphate) was due to pyruvate depletion. These results

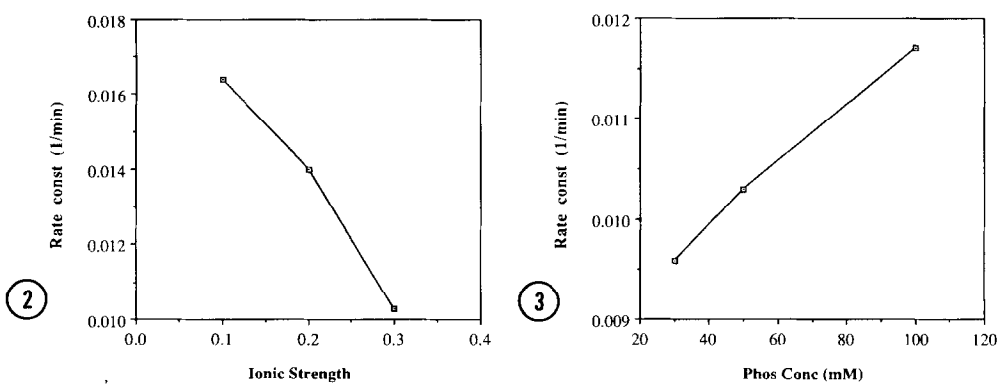


Fig. 2. Rate constant as a function of ionic strength. Reaction conditions were same as in Fig. 1,except ionic strength was varied with KCl. The buffer was 50 mM potassium phosphate (pH 7.0).

Fig.3. Rate constant as a function of phosphate concentration. Conditions were identical as in Fig. 1. The pH and ionic strength were kept at 7.0 and 0.3 respectively.

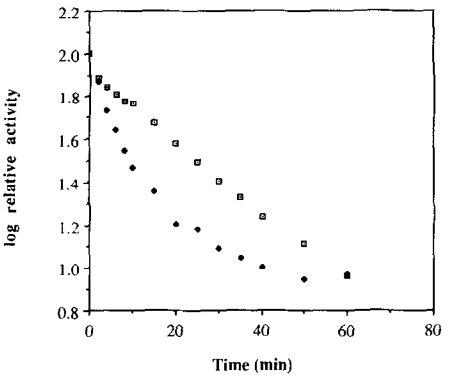


Fig. 4. Normalized inactivation profile (plotted on a semi-log scale) with 100 mM phosphate, (pH 7.5) (□), and with 50 mM MOPS + 0.021mM phosphate, (pH 7.5) (•). Conditions were otherwise identical as in Fig. 1.

indicate the involvement of phosphate in the reaction, although the specific nature of involvement is far from clear. It is possible that it might react with acetyl-TPP, which has been shown to be a transient species in the reactions catalyzed by PDH complex or its $E_1$ component (7), with $O_2$ as the electron acceptor.

Studies with [2-$^{14}$C]pyruvate

In order to study the reaction mechanism further, 2-$^{14}$C-labelled pyruvate was used as the incubating substrate, and was incubated with the enzyme for at least 35 min. The reaction products were latter separated by passing through a Sephadex G-25 column equilibrated with 5 mM phosphate buffer (pH 7.0). The fractions were subsequently counted for radioactivity and their absorbances at 280 nm were determined. No significant amount of radioactivity was found to be associated with the enzyme fractions. Our observations are similar to the findings by Williams *et al* (3).

In summary, we see a dependance of inhibition of PDH complex on pyruvate concentration. The rates are also dependant on the pH, ionic strengths and salt concentrations of the buffer. Our results indicate that the composition of buffer plays a role in the inhibition. The absence of any radio-labelled protein peak supports the hypothesis of Williams *et al.* (3) that formation of a covalent adduct was not necessary for strong inhibition, which is contradictory to the hypothesis proposed by Khailova *et al.* (1). Further experiments are needed to clarify the role of buffers in the inactivation reactions.

## ACKNOWLEDGMENT

I thank Prof. Perry A. Frey for allowing me to do this research in his laboratory, and also for many stimulating discussions.

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