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FLUOROPYRUVATE: AN UNUSUAL SUBSTRATE FOR ESCHERICHIA COLI PYRUVATE DEHYDROGENASE

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Summary: The pyruvate dehydrogenase component of the E. coli pyruvate dehydrogenase complex catalyzes the decomposition of 3-fluoropyruvate to acetate and fluoride ions in equimolar amounts and at about one-tenth the rate at which it catalyzes the conversion of pyruvate and ferricyanide to acetate and ferrocyanide. When the reaction is carried out in $[^3H]H_2O$ the product is [^{3}H]acetate. The reaction is strictly dependent upon added thiamin pyrophosphate, and a mechanistic role is proposed for this coenzyme.

In the course of our studies of the interactions of substrate analogues with the E. coli pyruvate dehydrogenase complex we have discovered a new reaction catalyzed by the pyruvate dehydrogenase (E_1) component of this complex. Pyruvate dehydrogenase is the TPP[1]-dependent component which catalyzes the decarboxylation of pyruvate in the presence of ferricyanide to produce acetate, ferrocyanide and CO_2 according to equation 1. The reaction is conveniently monitored by the absorbance

$$CH_3COCO_2^- + 2Fe(CN)_6^{-3} + H_2O \xrightarrow{E_1\cdot TPP} CO_2 + CH_3CO_2^- + 2Fe(CN)_6^{-4} + 2H^+ \quad (1)$$

decrease at 420 nm accompanying the consumption of ferricyanide. 3-Fluoropyruvate exhibits no detectable activity as an alternative substrate in equation 1, nor is it utilized as a substrate for the pyruvate dehydrogenase complex as a whole in the overall reaction, equation 2. In this

$$CH_3COCO_2^- + DPN^+ + CoA \xrightarrow{\text{PDH complex}} CO_2 + CH_3COCoA + DPNH \quad (2)$$

paper we show that pyruvate dehydrogenase catalyzes the TPP-dependent decomposition of 3-fluoropyruvate in a reaction which does not involve electron transfer to an oxidizing cosubstrate.

[1]The abbreviation is TPP, thiamin pyrophosphate.

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MATERIALS AND METHODS

The pyruvate dehydrogenase component of the pyruvate dehydrogenase complex was resolved and purified from the complex as described by Reed and Willms (1). The pyruvate dehydrogenase complex was purified by the procedure of Reed and Willms (1) as amended by Speckhard and Frey (2). Acetate kinase was purchased from Sigma Chemical Company.

Sodium fluoropyruvate was purchased from Sigma Chemical Company. That this material was suitable as supplied was verified by purifying a sample on a silicic acid column by the procedure described by Varner (3). 3-Fluoropyruvic acid was eluted with 10%-n-butanol in chloroform and, after removal of solvent by rotary evaporation, distilled at 70° and 4 mm pressure to produce a colorless crystalline solid melting at 58-60°. This sample was indistinguishable from the commercial sample in its interaction with pyruvate dehydrogenase. All other chemicals were purchased from commercial sources and used as supplied.

Pyruvate dehydrogenase was assayed by the ferricyanide procedure described by Maldonado et al. (4). Acetate production was monitored by a published procedure in which it is converted to acetohydroxamate and measured colorimetrically as the ferric acomplex (5). Radiochemical assays were carried out by liquid scintillation counting. [^{3}H]Acetic acid in silicic acid column fractions was measured by titrating the butanol-chloroform fractions with standard NaOH until the aqueous layers reached pH 5. Aliquots of the aqueous layers were then subjected to radiochemical analyses. Fluoride production was quantitatively measured by the use of an Orion combination fluoride electrode and an Orion digital pH/mV meter, Model 701A. Standard fluoride solutions were used to calibrate the electrode before each experiment.

RESULTS

In preliminary experiments we found that pyruvate dehydrogenase catalyzes the decomposition of 3-fluoropyruvate to fluoride and acetate ions and that the formation of both products is strictly dependent upon the presence of both pyruvate dehydrogenase and TPP (6). This suggested that fluoropyruvate may react as an alternative substrate according to equation 3. In this formulation fluoride and acetate are produced in equivalent

$$FCH_2COCO_2^- + H_2O \xrightarrow{E_1 \cdot TPP} CO_2 + CH_3CO_2^- + F^- + H^+ \qquad (3)$$

molar amounts and the source of one of the hydrogen atoms in acetate is H_2O. Table I confirms that fluoride and acetate are produced in equivalent amounts. The rate of the reaction under the conditions of Table I is about one-tenth that of the maximum rate at which the enzyme catalyzes equation 1.

The fluoride analysis in Table I is considered to be definitive because of the specificity of the fluoride electrode, however, the identifi-

Table I

Enzymatic Decomposition of Fluoropyruvate to Fluoride and Acetate

Time (min)	Fluoride (μmol/ml)	Acetate (μmol/ml)	Fluoride/Acetate
0	0	0	--
5	0.021	0.024	0.9
10	0.043	0.046	0.9
15	0.065	0.064	1.0
20	0.087	0.086	1.0
30	0.129	0.122	1.1

The reaction mixture consisted of 1 mM 3-fluoropyruvate, 1 mM TPP, 0.01 mM $MgCl_2$, 50 mM potassium succinate buffer at pH 6.7, and 1.8 units/ml of pyruvate dehydrogenase at 27°C. Aliquots were withdrawn at the indicated times and analyzed for acetate and fluoride as described under Methods. No trace of product could be detected in control experiments in which either TPP or enzyme was excluded from the reaction mixture.

cation of acetate as the color yielding product in the acetate assay is less certain. In this assay method acetate kinase catalyzes the phosphorylation of acetate by ATP to acetyl-phosphate, and hydroxylamine in the reaction mixture reacts with acetyl-phosphate to produce acetohydroxamate which is measured colorimetrically as the ferric complex. The specificity of acetate kinase for acetate is not absolute, so the identification of acetate in Table I is not definite.

In order to determine both whether this product actually is acetate and, if so, whether one of the hydrogens is derived from solvent protons as required by equation 3, the reaction was carried out in $[^3H]H_2O$. The reaction mixture consisted of 1 mM 3-fluoropyruvate, 1 mM TPP, 1 mM $MgSO_4$, 50 mM potassium phosphate buffer at pH 6.75, and 3.4 units/ml pyruvate dehydrogenase in $[^3H]H_2O$ having a specific radioactivity content of 4.2×10^4 cpm/μatomH. After 30 min at 29°C about 0.1 μmole of acetate had been produced and the solution was adjusted to pH 10 and heated to inactivate the enzyme. After adding 300 mg of carrier potassium acetate the $[^3H]H_2O$

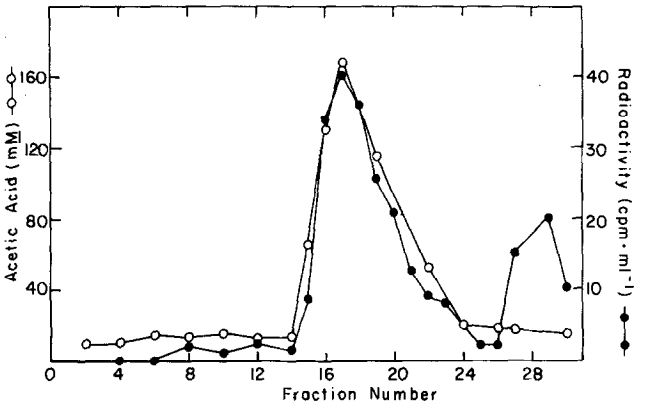

Figure 1: Silicic Acid Chromatography of [^{3}H]Acetic Acid

The reaction mixture and procedure for converting 3-fluoropyruvate to acetate in [^{3}H]H_2O are described in the text. After dilution with carrier acetate the [^{3}H]acetic acid was chromatographed on a 1 x 30 cm column of silicic acid (3) with elution by 2% 1-butanol in chloroform. The acetic acid and radioactivity contents of fractions were measured as described under Methods.

was removed by sublimation and the residue was subjected to silicic acid column chromatography (3). Analysis of collected fractions showed that a radioactive band coincided with the position of acetic acid against a very high background of radioactivity attributed to residual [^{3}H]H_2O. The acetic acid containing fractions were pooled and rechromatographed on an identical column. Again, as depicted in Figure 1, a radioactive band was eluted in exactly the position of acetic acid. The specific radioactivity of acetic acid was 0.73×10^4 cpm/μmole after correction for dilution effects. When compared with the specific activity of the [^{3}H]H_2O used this corresponded to an isotopic discrimination against tritium relative to protium by a factor of about 6.

DISCUSSION

The conversion of 3-fluoropyruvate to acetate by this enzyme in the absence of ferricyanide can be related to the decomposition of pyruvate to acetate in the presence of ferricyanide. In the first place 3-fluoropyruvate is at a two electron higher oxidation state than pyruvate, so that an added

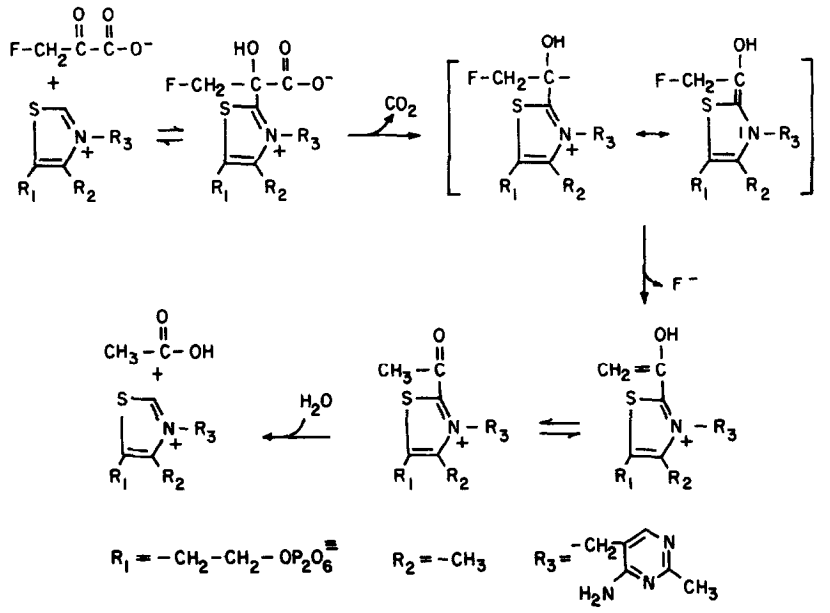

Figure 2: Proposed Role of TPP in Enzymatic Reaction of 3-Fluoropyruvate

electron acceptor is not required, and secondly a reasonable mechanistic pathway can be proposed based on the chemical properties of TPP and the current understanding of the mechanisms of TPP action in enzymatic reactions (7,8). In Figure 2 we propose that TPP associated with pyruvate dehydrogenase initially interacts with 3-fluoropyruvate essentially as it does with pyruvate, by forming an adduct and then promoting the decarboxylation of the adduct. The resulting α-hydroxy-β-fluoro-ethylidene-TPP eliminates fluoride ion, and the resulting enol tautomerizes to acetyl-TPP with incorporation of solvent hydrogen at acetyl-C-2. Finally, acetyl-TPP undergoes hydrolysis to acetic acid and TPP. The elimination of fluoride ion in Figure 2 is analogous to the dehydration of α,β-dihydroxyethyl-TPP catalyzed by phosphoketolase. Whether pyruvate dehydrogenase actively catalyzes fluoride elimination or whether rapid elimination of fluoride is an intrinsic property of the intermediate cannot be decided at this time.

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