ACETYLATION STOICHIOMETRY OF ESCHERICHIA COLI PYRUVATE DEHYDROGENASE COMPLEX

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Summary: [1-^{14}C]Acetyl-pyruvate dehydrogenase complex can be isolated either by gel filtration or by phenol extraction upon reaction of the complex with [2-^{14}C]pyruvate in the absence of coenzyme A and NAD. Acetylation by [2-^{14}C]pyruvate is thiamine pyrophosphate dependent, all of the radioactivity is covalently bonded to the dihydrolipoyl transacetylase component, and all of the [1-^{14}C]acetyl groups are removed by coenzyme A. The radioactivity incorporated corresponds to 9.6 to 10.1 n·mol of acetyl groups per mg dry weight of complex, about four times the number of FAD molecules. Based on a particle molecular weight of 4.6 to 4.8 x 10^6, this stoichiometry corresponds to 48 acetyl-group binding sites and 12 molecules of FAD per particle of complex.

The E. coli pyruvate dehydrogenase complex consists of three enzymes which act in coordination to catalyze the conversion of pyruvate, NAD^+, and CoA to CO_2, acetyl-CoA and NADH (1). They are pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase, designated respectively E_1, E_2, and E_3. The molecular weight is about 4.6 to 4.8 million (2), and E_2, which contains covalently bonded α-lipoic acid, is the core to which the other two enzymes are bound (3,4).

Equations 1-5 set forth the proposed catalytic pathway for oxidative decarboxylation of pyruvate by this complex (5,6).

$$H^+ + E_1\cdot TPP^1 + CH_3COCO_2^- \rightarrow CO_2 + E_1\cdot \text{hydroxyethyl-TPP} \quad (1)$$

$$E_1\cdot \text{hydroxyethyl-TPP} + \text{lipoyl-}E_2 \rightarrow E_1\cdot TPP + \text{S-acetyldihydrolipoyl-}E_2 \quad (2)$$

$$\text{S-acetyldihydrolipoyl-}E_2 + CoA \rightleftharpoons \text{Acetyl-CoA} + \text{dihydrolipoyl-}E_2 \quad (3)$$

$$\text{dihydrolipoyl-}E_2 + E_3\cdot FAD \rightleftharpoons \text{lipoyl-}E_2 + \text{dihydro-}E_3\cdot FAD \quad (4)$$

$$\text{dihydro-}E_3\cdot FAD + NAD^+ \rightleftharpoons E_3\cdot FAD + NADH + H^+ \quad (5)$$

SUM: $CH_3COCO_2^- + NAD^+ + CoA \rightarrow CO_2 + \text{Acetyl-CoA} + NADH$

[1]The abbreviation is TPP, thiamin pyrophosphate

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The intermediates have heretofore not been characterized within the complex itself, although the redox mechanism of purified E_3·FAD has been investigated (6,7).

In this paper we report the isolation of the acetylated complex and the stoichiometry of acetyl group binding.

MATERIALS AND METHODS

Pyruvate dehydrogenase complex was purified from E. coli ATCC 8739 (Crookes strain), or from Strain K-12 purchased from Grain Processing Corp., Muscatine, Ia., by the published procedure (8) as modified and extended to include chromatography on calcium phosphate gel-cellulose (9). The specific activities of all preparations were 37-45 μmol NADH per min per mg of protein under the assay conditions of Maldonado et al. (10).

Sodium [2-^{14}C]pyruvate and [1-^{14}C]pyruvate were obtained from New England Nuclear or Amersham-Searle and diluted with carrier. The specific radioactivity of [^{14}C]pyruvate in solution was measured as follows. The [pyruvate] was measured spectrophotometrically as NADH in the presence of pyruvate dehydrogenase, NAD^+ and thiamin pyrophosphate. A sample of known ^{14}C content was diluted into an accurately measured sample of pure carrier pyruvate and converted to the semicarbazone. The specific radioactivity content of pyruvate in the original solution was calculated from the measured specific radioactivity of the solid derivative, after correcting for isotope dilution.

Protein concentrations were measured by the Lowry method, using crystalline bovine serum albumin as the standard, or by measurement of A_{280}. Both methods were correlated with dry weight of pyruvate dehydrogenase complex and all protein analyses were corrected to dry weight. Radiochemical analyses were made by liquid scintillation counting in a Packard model 3310 TriCarb spectrometer.

RESULTS

According to equations 1 and 2 pyruvate should acetylate the complex with thiamin pyrophosphate dependence in the absence of CoA. The results of acetylation experiments with [^{14}C]pyruvate are given in Table I, and they are in essential accord with the proposed pathway.

Two methods were used to isolate the acetylated complex, gel filtration and phenol extraction. In experiments 1 through 4, 1.0 to 3.0 mg of complex were combined with 0.1 to 0.3 μmol [^{14}C]pyruvate, 1 mM $MgSO_4$, 0.2 mM thiamin pyrophosphate, and 50 mM potassium phosphate buffer at pH 7.0 in a total volume of 0.2 to 0.6 ml at 4°C. The solutions were immediately passed through columns of Sephadex G-25 at 4°C, and those fractions in the excluded

Table I

Acetylation of Pyruvate Dehydrogenase Complex by [2-^{14}C]pyruvate

Experiment	Acetylation Conditions	Isolation	^{14}C-Incorporated (n·mol/mg complex)
1	KP_i, pH 7.0, 4°	gel filtration	9.6 ± 0.3
2	[1-^{14}C]pyruvate	gel filtration	0.03 ± 0.02
3	+ CoA + NAD^+	gel filtration	0.03 ± 0.01
4	thiamin pyrophosphate omitted	gel filtration	0.03 ± 0.01
5	Na-bicinate, pH 8.1, 27°	phenol extraction	10.1 ± 0.7
6	1 min as Expt 5 then excess CoA + NAD^+	phenol extraction	0.56 ± 0.3

The labeling procedures and complete reaction mixture compositions are given in the text. In experiment 2, [2-^{14}C]pyruvate was replaced with [1-^{14}C]pyruvate. In experiment 3, CoA and NAD^+ in large excess were included in the reaction mixture. In experiment 6, the acetylation procedure of experiment 5 was carried out for 1 min; then excess CoA and NAD^+ were added, and the reaction was quenched with phenol 1 min later.

volumes were subjected to radiochemical and protein analyses. There was no significant difference in ^{14}C-incorporation between commercially supplied [2-^{14}C]pyruvate, which contained variable amounts of radiochemical impurities, and [2-^{14}C]pyruvate that had been purified by silicic acid chromatography (11).

Experiment 5 is a compilation of data from many repetitions of labeling experiments in Na-bicinate buffer at pH 8.1 and 27°, in which the denatured [1-^{14}C]acetylated complex was isolated by extraction into phenol. The reaction mixtures contained 0.25M Na-bicinate buffer at pH 8.1, 1.25 mM $MgSO_4$, 1.25 mM thiamin pyrophosphate, 0.21 mg/ml pyruvate dehydrogenase complex and 0.004 to 1.97 mM [2-^{14}C]pyruvate in a total volume of 0.2 ml at 25°C. [2-^{14}C] Pyruvate was added last and, after 1 min, 1.0 ml of water-saturated phenol was added. The phenol layers were repeatedly washed with phenol-saturated water until the washes contained no significant radioactivity. Then aliquots of the phenol layers were subjected to radiochemical analysis. In experiment 6 the

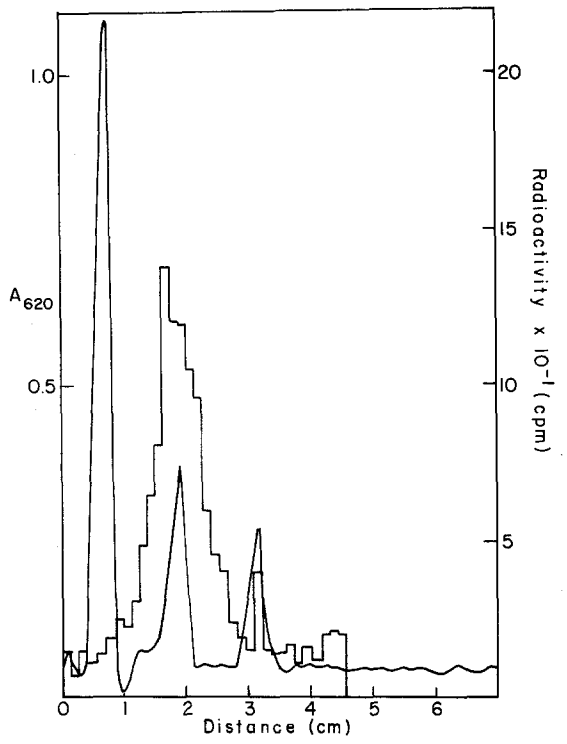

Figure 1: Sodium Dodecyl Sulfate Polyacrylamide Disc Gel Electrophoresis of [1-^{14}C]acetyl-pyruvate Dehydrogenase Complex.

A sample of complex was acetylated with [2-^{14}C]pyruvate under the conditions of experiment 5 in Table I and then precipitated with trichloroacetic acid in the cold. The precipitate was washed with cold trichloroacetic acid and ether, and then dissolved in 10 mM sodium phosphate buffer at pH 7.0 containing 6M urea and 1% sodium dodecyl sulfate. Samples were subjected to polyacrylamide disc gel electrophoresis on 12% polyacrylamide gels cast in the above buffer. One gel was strained with comassie brilliant blue and scanned at 620 nm to locate the protein. A second was sliced into 1.4 mm sections, which were subjected to radiochemical analysis after solubilization with Protosol. The continuous tracing in the figure is A_{620} and the step-graph depicts the radiochemical data. The fastest moving protein is dihydrolipoyl dehydrogenase, the middle protein is dihydrolipoyl transacetylase, and the slowest is the pyruvate dehydrogenase component.

same procedure was followed with 0.004 mM [2-^{14}C]pyruvate, except that 0.25 to 1.25 mM CoA and 2.5 to 12.5 mM NAD^+ were added after the 1 min labeling period and the solutions were quenched with phenol an additional 1 min later.

The results of these experiments show that about 10 n·mol of acetyl groups are incorporated per mg of complex with thiamine pyrophosphate dependence, and

essentially all are CoA-labile. This is at least four times the FAD content of the complex (9).

Figure 1 gives the results of a sodium dodecyl sulfate polyacrylamide disc gel electrophoresis experiment on the complex acetylated with [2-^{14}C]pyruvate. It clearly shows that essentially all the acetyl groups are bonded to dihydrolipoyl transacetylase, the core protein in the complex, presumably to the dihydrolipoyl moieties bonded to this protein.

DISCUSSION

The data in Table I were obtained from numerous preparations of E. coli pyruvate dehydrogenase complex purified from both Crookes strain and K-12. We have never detected significant variation from one preparation to another, which clearly shows that the complex is a definite, highly reproducible entity.

The FAD content is 1.9 to 2.5 n·mol per mg of complex (9) and all the FAD is reducible by substrates, either pyruvate in the presence of thiamin pyrophosphate and CoA or NADH alone (12). Assuming the molecular weight is 4.6 to 4.8 million (2) our data show that there are 11.5 to 12 functional FAD molecules in each particle and 46 to 48 sites potentially able to accept a pair of electrons and an acetyl group. All of the latter are associated with the dihydrolipoyl transacetylase component. The data are compatible with the subunit composition model proposed by Reed et al. (4) which is that the E_1:E_2:E_3 chain ratios are 24:24:12. Our results show, however, that there must be two acetyl group and electron pair acceptor sites per transacetylase chain. These are presumably α-lipoyl moieties. Other data also suggest more than one α-lipoic acid per chain (13).

Our acetylation experiments establish the presence of at least four acetyl group and electron acceptor sites in E_2 per FAD in E_3. Inasmuch as these experiments were performed under conditions contrived to arrest enzyme action at the acetylation stage rather than under conditions of catalysis of the overall reaction, they do not bear on the question of whether all the

acetylation sites are mechanistically coupled to FAD sites and, if so, by what mechanism. These questions remain for further investigation.

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