ESHERICHIA COLI PYRUVATE DEHYDROGENASE COMPLEX: IMPROVED PURIFICATION AND THE FLAVIN CONTENT

David C. Speckhard* and Perry A. Frey
Department of Chemistry, The Ohio State University
Columbus, Ohio 43210

Received December 9,1974

Summary: The E. coli pyruvate dehydrogenase complex, when purified by published procedures, contains phosphotransacetylase and coenzyme A as trace contaminants as well as one or more spectral contaminants which interfere with spectral and radiochemical experiments. They can be removed by further chromatographic purification on columns of calcium phosphate gel-cellulose. The resulting complexes from E. coli K12 or Crookes strain are indistinguishable with respect to visible spectrum, catalytic activity, and flavin content. The activity is the highest so far reported, 40-42 μmoles DPNH per min per mg of protein, and the flavin content is 1.8-2.4 nanomoles per mg of protein.

The pyruvate dehydrogenase complex from Escherichia coli, first purified by Reed and coworkers (1), consists of three enzymes: pyruvate dehydrogenase, dihydrolipoyl transacetylase, and the flavoprotein dihydrolipoyl dehydrogenase. Two stoichiometry models recently proposed differ both in the proposed number of each enzyme molecule in the complex and in the ratios of those numbers. One postulates that each particle of complex contains 12 molecules of the flavoprotein and 24 molecules of each of the other two (2). The other postulates that each particle consists of 16 molecules of each of the three enzymes (3). The models are based on different kinds of analytical data obtained on preparations of the complex purified by different methods from different strains of E. coli, Crookes strain in one case (2) and regulatory mutants of K12 in the other (3). Some of the data on which the two models are based cannot be directly compared. The direct comparisons that can be made are the molecular masses of the component enzymes, which are essentially the same, and the measured molecular masses of the purified complexes, which are different (2,3,4). One other possible comparison is the flavin content, which is reported by Eley, et al. to be 2.6 to 2.8 nanomoles per mg of protein (2) and by Vogel, et al. to be 4.4 nanomoles per mg of protein (3). The flavin content is a key factor used by both groups in arriving at their interpretive models.

*Allied Chemical Company predoctoral fellow, 1973-1974.

In this paper we describe an improved purification procedure, which extends the original procedure and produces an improved complex, and we also show that the complexes purified from Crookes strain and from K12 by this procedure are identical with respect to catalytic activity, visible spectrum, and flavin content.

MATERIALS AND METHODS

Materials--We purified pyruvate dehydrogenase complex from Escherichia coli K12 cells purchased from Grain Processing Corporation, Muscatine, Iowa, and from Crookes strain cells, ATCC 8739 grown in our laboratory, as described by Reed and Mukherjee with one exception (5). We modified the isoelectric fractionation step, which is the last step of the procedure and which separates pyruvate dehydrogenase complex from α-ketoglutarate dehydrogenase complex. The procedure of Reed and Mukherjee called for a pH adjustment to 5.7 to precipitate α-ketoglutarate dehydrogenase complex followed by further lowering the pH of the supernatant fluid to 4.9 to precipitate pyruvate dehydrogenase complex. In our modification we collected the precipitates at pH's 5.7, 5.4, 5.1 and 4.9. In this way we almost always achieved satisfactory separations of the two complexes. The original procedure frequently failed in our hands, apparently due to a degree of variability in the behavior of these complexes from one preparation to another.

Dihydrolipoyl dehydrogenase was purified from Escherichia coli α-ketoglutarate dehydrogenase complex as described (6).

Methods--Protein concentrations were measured by the method of Lowry et al. (7) using anhydrous crystalline bovine serum albumin as the standard. The flavin content of the complex was measured by first dissociating the FAD by trichloroacetic acid precipitation of the protein and then recording the absorbance decrease at 450 nm of the neutralized supernatant fluid upon reduction with dithionite (8). Pyruvate dehydrogenase complex activity was measured as the rate of DPNH appearance at pH 8.1 and 27° with optimal substrate and cofactor concentrations (9). Under otherwise similar assay conditions but at pH 7.0 (10) we found the activity of the complex at 27° to be the same as at pH 8.1.

RESULTS AND DISCUSSION

During the course of radiochemical experiments to be described elsewhere we found the Escherichia coli pyruvate dehydrogenase complex to be contaminated with phosphotransacetylase and coenzyme A when purified by the published procedure (5) either from commercially grown K12 cells or from Crookes strain cells. Phosphotransacetylase contamination amounted to 0.2 to 5 units per mg of protein when assayed as described by Bergmeyer et al. (11), while the coenzyme A contamination was estimated to be on the order of enzyme concentration. We also detected variable amounts of spectral contamination in the visible spectrum of the complex purified from either strain.

These contaminants are removed by further chromatographic purification. At the same time the specific activity of the complex is increased by a factor of up to two. Data from a typical chromatographic purifi-

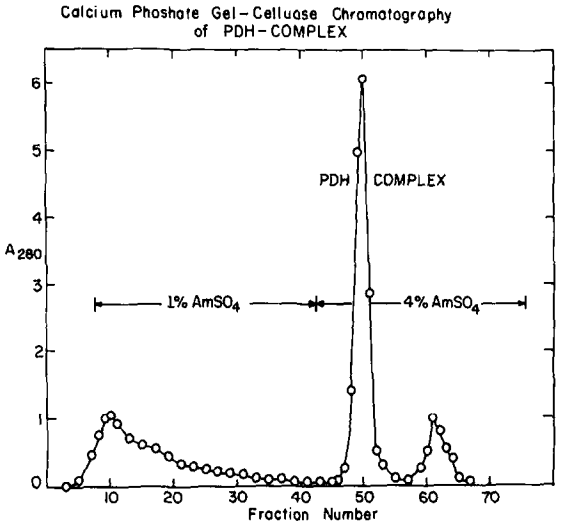

Figure 1: Calcium phosphate gel-cellulose column chromatography of pyruvate dehydrogenase complex. Calcium phosphate gel adsorbed on cellulose is prepared as described by Reed and Willms (12) and used to prepare a 2.8 x 5 cm gravity packed column at room temperature. After temperature equilibration at 4°, up to 200 mg of pyruvate dehydrogenase complex, obtained from isoelectric fractionation as described under Methods, at 5 mg/ml in 50 mM potassium phosphate buffer at pH 7.0 is permitted to flow into the column by gravity flow. The initial flow rate should be 15 ml per hour with a 10 to 15 cm hydrostatic head. The column is then washed with 1% $(NH_4)_2SO_4$ in 100 mM potassium phosphate buffer at pH 7.5 at a flow rate of 15 ml per hour until the absorbance of the effluent at 280 nm approaches zero. The complex is then eluted at the same flow rate with 4% $(NH_4)_2SO_4$ in the same buffer.

---

cation on a column of calcium phosphate gel-cellulose are given in Figure 1. The complex itself is strongly adsorbed from 50 mM potassium phosphate buffer at pH 7, but a substantial amount of protein is eluted in the flow through and the 1% $(NH_4)_2SO_4$ wash. This contains the phosphotransacetylase activity and any contaminating α-ketoglutarate dehydrogenase complex. The pyruvate dehydrogenase complex is then eluted in excellent yield as a sharp band with 4% $(NH_4)_2SO_4$. When spectral contamination is substantial, a red band eluting just behind the complex can *sometimes* be detected visually.

In Figure 1 the overall recovery of protein was 85%, the recovery of pyruvate dehydrogenase complex activity was 90%, and its specific activity increased from 19 to 41 units per mg of protein. The complex purified in this way invariably gave three sharp bands when subjected to sodium dodecylsulfate polyacrylamide disc gel electrophoresis, with no significant contamination. Quite significant contamination was detected by this method when applied prior to chromatography.

Figure 2 shows the visible spectra of three preparations of E. coli pyruvate dehydrogenase complex. That purified from commercially grown K12 strain through the isoelectric fractionation exhibits definite contamination at 420 nm and also in the 500-650 nm region. This spectrum is typical although there is variability in the degree of contamination among different preparations. The published spectrum of the complex purified from Crookes strain by the same procedure exhibits less contamination (1). We also find less in our preparations purified from Crookes strain grown under the same conditions; however, there is invariably at least a plateau in the 420 nm region and usually a detectable band. As shown in Figure 2 this contamination is removed by chromatography on

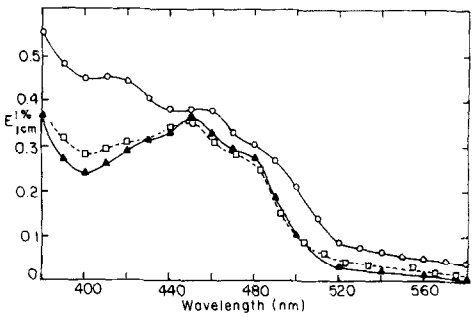

Figure 2: Visible spectra of pyruvate dehydrogenase complex. The spectra were recorded with a Norelco/Unicam SP800A double beam spectrophotometer at protein concentrations between 6 and 12 mg per ml and normalized to 10 mg per ml. Symbols: O—O, pyruvate dehydrogenase complex purified from E. coli K12 as described (5); □—□, the same complex after chromatography as described in the legend to Figure 1; ▲—▲, a preparation of the complex purified from Crookes strain as described in Figure 1.

calcium phosphate gel-cellulose columns, and the spectra of complexes from both strains are essentially the same. Although the two spectra of chromatographed complexes in Figure 2 are not quite identical they do not differ by more than the spectra of any two preparations from the same strain.

As suggested by their spectral identity, and in agreement with their identical catalytic activities, preparations of this complex from the two strains have the same flavin content, 1.8 to 2.4 nanomoles per mg of protein, as determined spectrophotometrically by reducing the flavin adenine dinucleotide with dithionite after releasing it from the complex by acid treatment. We consider this to be a minimum estimate of the

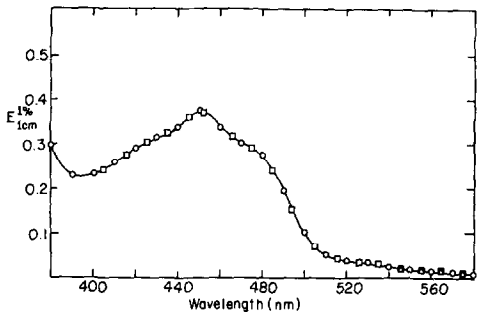

Figure 3: Visible spectrum of pyruvate dehydrogenase complex before and after incubation with dihydrolipoyl dehydrogenase. The experimental protocol is given in the text. Symbols: O—O, spectrum prior to incubation with flavoprotein; □ — □, spectrum following reisolation of the complex from flavoprotein.

true flavin content; 2.4 n·moles per mg of protein is probably near the correct value, and it approaches the 2.6-2.8 nanomoles per mg of protein reported by Eley et al. (2) but is much smaller than the 4.4 nanomoles per mg of protein reported by Vogel et al. (3).

We have considered the possibility that our preparations of pyruvate dehydrogenase complex purified by chromatography might be deficient in the flavoprotein, dihydrolipoyl dehydrogenase. However, in repeated attempts we have been unable to show that our preparations will bind or be activated by additional purified dihydrolipoyl dehydrogenase. In one experiment, for which we have the most complete data, we measured the spectrum of a typical preparation of the complex, then incubated 19 mg of this complex with 1.3 mg of purified dihydrolipoyl dehydrogenase in 2.6 ml of 20 mM potassium phosphate buffer at 25° and pH 7.0. In a control experiment 1.0 mg of the same complex was incubated in the absence of dihydrolipoyl dehydrogenase under otherwise identical conditions. Pyruvate dehydrogenase complex activities in the two solutions were measured periodically over a period of 1.5 hr at 25°. We found no difference between them. We then reisolated the complex by preparative ultracentrifugation, which sedimented the complex but not dihydrolipoyl dehydrogenase. The liquid pellet was redissolved in the same buffer and again subjected to ultracentrifugation to ensure that all traces of free dihydrolipoyl dehydrogenase would be removed. The pellet was again redissolved and its spectrum was measured. As shown in Figure 3, we found its spectrum to be identical with that recorded prior to incubation with dihydrolipoyl dehydrogenase.

This complex also appears to contain a small amount of thiamine pyrophosphate in latent form. In ordinary activity assays the action of the complex is thiamine pyrophosphate dependent, however, if thiamine pyrophosphate is omitted from the standard assay solution, a larger than normal amount of enzyme complex will produce a significant rate. The rate appears gradually during a lag period of 5 to 15 minutes and finally reaches a zero order rate of 2 to 5% of the maximum thiamine pyrophosphate dependent initial rate. The lag can be abolished by preliminary incubation of the complex with pyruvate for 15 to 20 minutes and then completion of the reaction mixture with coenzyme A and DPN^+. This apparent latent thiamine pyrophosphate is not removed by chromatography under the conditions of Figure 1.

In their recent studies Eley et al. (2) purified the pyruvate dehydrogenase complex by a different modification of the procedure of Reed and Mukherjee (5). Their modified procedure also produced an improved complex with a specific activity of about 35 units per mg of protein, nearly as high as the 40-42 units per mg of protein reported here for chromatographically purified complex.

In conclusion, calcium phosphate gel-cellulose column chromatography of the pyruvate dehydrogenase complex described by Koike et al. (1) removes contaminating phosphotransacetylase, coenzyme A and one or more trace spectral contaminants. With respect to catalytic activity, visible spectrum, and flavin content the complexes from Crookes strain and K12 strain E. coli are indistinguisable, and the flavin content is more consistent with the data of Eley et al. (2) than with those of Vogel et al. (3).

Acknowledgment

This work was supported by Grant No. AM 13502 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

References

1. Koike, M., Reed, L.J., and Carroll, W.R. (1960) J. Biol. Chem. 235, 1924-1930.
2. Eley, M.H., Namihira, G., Hamilton, L., Munk, P., and Reed, L.J. (1972) Arch. Biochem. Biophys. 152, 655-669.
3. Vogel, O., Hoehn, B., and Henning, V. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 1615-1619.
4. Vogel, O., Hoehn, B., and Henning, V. (1972) Eur. J. Biochem. 30 354-360.
5. Reed, L.J. and Mukherjee, B.B. (1969) Methods in Enzymology 13, 55-61.

6. Pettit, F.H., Hamilton, L., Munk, P., Namihira, G., Eley, M.H., Willms, C.R., and Reed, L.J. (1973) J. Biol. Chem. 248, 5282-5290.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Beinert, H. and Page, E. (1957) J. Biol. Chem. 225, 479-497.
9. Maldonado, M.E., Oh, K-J., and Frey, P.A. (1972) J. Biol. Chem. 247, 2711-2716.
10. Moe, O.A. Jr. and Hammes, G.G. (1974) Biochemistry 13, 2547-2552.
11. Bergmeyer, H.U., Holz, G., Klotzsch, H., and Lang, G. (1963) Biochem. Z. 338, 114-121.
12. Reed, L.J. and Willms, C.R. (1966) Methods in Enzymology 9, 247-265.