Pyruvate Decarboxylase

I. PROTEIN DISSOCIATION INTO SUBUNITS UNDER CONDITIONS IN WHICH THIAMINE PYROPHOSPHATE IS RELEASED*

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ANNE D. GOUNARIS,† IRIS TURKENKOPF, SHARON BUCKWALD,‡ AND ANNE YOUNG¶

From the Department of Chemistry, Vassar College, Poughkeepsie, New York 12601

SUMMARY

Pyruvate decarboxylase, isolated from active dry baker's yeast, dissociates into subunits of one-half the molecular weight at alkaline pH. This has been determined by chromatography on Sephadex G-200 over the pH range 6.5 to 8.0. The conditions at which protein dissociation is observed are the same as the conditions at which the cofactors thiamine pyrophosphate and Mg²⁺ are released and can be separated from the protein.

When the holoenzyme is reconstituted in the presence of thiamine-PP and Mg²⁺ the active enzyme is a dimer of the same molecular weight as the original native enzyme. When the apoenzyme, subjected to the reconstitution procedure in the absence of thiamine-PP and Mg²⁺, is chromatographed on Sephadex G-200 at pH 6.5 the protein is eluted at an intermediate position, suggesting a monomer-dimer equilibrium which favors the monomer.

It appears that thiamine-PP in addition to its catalytic role also functions in the formation of a stable dimer which is the active holoenzyme. The presence of a subunit association step in the reconstitution process which requires thiamine-PP and Mg²⁺ identifies a site which may be related to the unusual requirement for excess thiamine-PP in the reconstitution process.

A method for selectively staining pyruvate decarboxylase on disc electrophoresis gels with a fuchsin staining reagent is described.

Yeast cytoplasmic pyruvate decarboxylase (2-oxo-acid carboxy-lyase, EC 4.1.1.1) catalyzes the decarboxylation of pyruvic acid producing acetaldehyde and carbon dioxide. Thiamine pyrophosphate and magnesium ion are obligatory cofactors for this reaction (1-3). Steyn-Parve and Westenbrink (4) reported that at pH 8.0 thiamine-PP⁴ and presumably Mg²⁺ were released forming apoenzyme as depicted by Equation 1.

Holoenzyme → apoenzyme + thiamine-PP + Mg²⁺ (1)

These findings have been confirmed by other investigators (3, 5, 6). Although the reconstituted enzyme appeared to be identical with the original enzyme the reconstitution process exhibited some rather unexpected properties. When the same amount of thiamine-PP was added to the reconstitution mixture as had been originally present a small amount of activity was recovered. The presence of a large excess of thiamine-PP is required to recover total activity (4). Similar inconsistencies between the amount of thiamine-PP incubated with enzyme and extent of activity recovered have been reported (3, 5). Schellenberger has entitled the failure of binding of thiamine-PP to enzyme when sufficient coenzyme is available as the "quasi-irreversibility of the bonding between coenzyme and apoenzyme." In 1966 Schellenberger and Hübner (7) proposed the following mechanism for the reconstitution process:

\[
\text{Apoenzyme + thiamine-PP + Mg²⁺ → enzyme (2)}
\]

\[
\text{Enzyme → apoenzyme + thiamine-PP (3)}
\]

In this mechanism Equation 2 represents a fast nonordered addition of thiamine-PP and Mg²⁺ to form an inactive ternary complex. Equation 3 represents a slow cyclization of the enzyme-bound thiamine-PP and Mg²⁺ to form active enzyme. Schellenberger has suggested that the "quasi-irreversibility" of coenzyme binding is explained by a kinetically controlled equilibrium of Equation 3 since the concentration of the inactive ternary complex is determined by the equilibrium constants for the binding of thiamine-PP and Mg²⁺ to apoenzyme indicated by Equation 2.

In 1966 Ulrich, Wittorf, and Gubler (8) reported that the pyruvate decarboxylase isolated from brewer's yeast had a

* The abbreviation used is: thiamine-PP, thiamine pyrophosphate.
molecular weight of 175,000. Considering the size of the protein, they looked for but did not detect evidence of protein dissociation in the presence of urea. Recently, Ulrich and Kempel (9) have reported that, after denaturation accomplished by incubating pyruvate decarboxylase in 6 M guanidine hydrochloride for 2 to 4 days at room temperature with β-mercaptoethanol, the protein dissociated into subunits of about 90,000 molecular weight.

In this investigation evidence has been obtained which indicates that the active enzyme dissociates into subunits at pH 8.0. Chromatographic behavior of the pyruvate decarboxylase on Sephadex G-200 over the pH range 6.5 to 8.0 is presented. The subunit is one-half the molecular weight of the original active enzyme. This protein dissociation, predominantly a function of pH, is also affected by the buffer species. Protein dissociation occurs under the same conditions that thiamine-PP is released from the enzyme. The results indicate that reconstitution of the holoenzyme involves a subunit association step. The formation of active dimer requires the presence of thiamine-PP. Therefore, in addition to its catalytic role in the mechanism of the reaction (5, 10–16), it is proposed that thiamine-PP also functions in the formation of a stable enzymatically active dimer. This suggests that the characteristics of this association step may eventually provide an alternative explanation for the unusual properties of the reconstitution process.

**METHODS AND MATERIALS**

**Reagents**—Active bakers' yeast, type 1821, donated by Standard Brands, Inc., was the source of the pyruvate decarboxylase. DEAE-cellulose was purchased from Bio-Rad. The molecular weight calibration kit containing the enzymes aldolase, ovalbumin, chymotrypsinogen A, and RNase A, and the Sephadex resins were obtained from Pharmacia. Thiamine pyrophosphate, sodium pyruvate, yeast alcohol dehydrogenase, NADH, L-cysteine HCl, and bovine serum albumin were purchased from Sigma.

**Enzyme Preparation**—Pyruvate decarboxylase was isolated from bakers' yeast according to the procedure of Ulrich et al. (8) with the modifications indicated. The dry yeast was pulverized in a Waring Blender at high speed for 3 min in the cold room. The 5% glycerol solution contained 5 mM L-cysteine HCl and was adjusted to pH 6.5 with KOH. The precipitate obtained at 50 to 60% ammonium sulfate saturation was chromatographed on DEAE-cellulose. The precipitate was washed with 150 mM KCl and 0.01 M potassium phosphate, pH 6.3. A column, 30 x 1.5 cm, was packed with the slurry and equilibrated with 0.025 M potassium phosphate and 0.12 mg of yeast alcohol dehydrogenase in a total volume of 0.2 liter. The enzyme was first incubated for 2 min at 4°C in 0.1 M potassium phosphate buffer (pH 6.2), 33 μmoles of sodium pyruvate, 0.5 μmole of NADH, 1.5 μmoles of thiamine-PP, and 5 mM L-cysteine HCl, and 5 mM L-cysteine HCl was added to adjust pH 6.3. The column was equilibrated for 72 hours with a solution of 0.02 M potassium phosphate-0.1 M KCl, pH 6.5. Mg++, thiamine-PP, and L-cysteine HCl were not added to the solutions used for Sephadex G-200 chromatography. A flow rate of 8 ml per hour was maintained with a 10- to 12-cm hydrostatic pressure head. A solution of pyruvate decarboxylase in potassium phosphate buffer, pH 6.3, was applied and 3.0-ml fractions were collected. The protein was monitored by optical density at 280 μm. Additional Sephadex G-200 chromatograms were carried out with various buffer solutions described below.

**Protein Determination**—Protein concentration of the crude extract was measured by the Biuret method (17, 18). Bovine serum albumin was used for preparing the standard curve. Following extraction from the acetone precipitate, the protein concentration was estimated by the method of Warburg and Christian (19).

**Enzyme Activity**—Pyruvate decarboxylase activity was determined by an assay in which the decarboxylase activity was coupled with alcohol dehydrogenase (8). The oxidation of NADH was followed at 340 μm with a Gilford model 240 spectrophotometer attached to a Photovolt Varicord model 43 recorder. The reaction mixture contained 225 μmoles of sodium succinate buffer (pH 6.2), 33 μmoles of sodium pyruvate, 0.5 μmole of NADH, and 0.12 mg of yeast alcohol dehydrogenase in a total volume of 3.0 ml. The enzyme was first incubated for 2 min at 4°C in 0.1 M potassium phosphate, pH 6.3, containing 25 μmoles of MgSO4 and 1.5 μmoles of thiamine-PP in a total volume of 1.0 ml before initiating the reaction. All assays were carried out at 25°C. Enzyme activity is expressed as micromoles of NADH oxidized per min per mg of protein.

**Disc Acrylamide Electrophoresis**—Disc electrophoresis was performed by the method described by Davis (20). The gels were prepared as described in the Canalco manual and the cell compartments were filled with a solution of 0.15 M Tris-0.15 M glycine, pH 8.9. Then 1.25 mA of current per tube were applied until the tracking dye migrated to the stacking gel and then was increased to 2.5 mA per tube for the remaining period. All runs were carried out at 25°C in a Buchler Polyanalyst No. 3 electrophoresis apparatus. Amido black was used for detecting the protein bands. Pyruvate decarboxylase activity was detected with a fuchsin staining reagent developed for this work.

**Detection of Pyruvate Decarboxylase Activity with Fuchsin Staining Reagent**—Fuchsin reagent was prepared as described by...
Purification of pyruvate decarboxylase from baker's yeast

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume ml</th>
<th>Total protein mg</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>3,850</td>
<td>40,922</td>
<td>0.46</td>
<td>100</td>
<td></td>
<td></td>
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<tr>
<td>Acetone extract</td>
<td>7,029</td>
<td>15,065</td>
<td>2.57</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate precipitate, 50-60%</td>
<td>83</td>
<td>1,354</td>
<td>8.42</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose Peak I</td>
<td>16</td>
<td>33.4</td>
<td>30.2</td>
<td>65.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose Peak I recombinantographied</td>
<td>25.4</td>
<td>5.31</td>
<td>180.9</td>
<td>34.1</td>
<td>74.3</td>
<td></td>
</tr>
</tbody>
</table>

*Specific activity = micromoles of NADH oxidized per min per mg of protein.

Therefore, one can identify the pyruvate decarboxylase on the separating gel and correlate this band with the appropriate protein band on a duplicate separating gel stained with Amido black. Following electrophoresis, the separating gel is immersed in the fuchsin staining reagent. Within 10 to 15 min the solution is colorless and the reagent must be replaced. From this time approximately 2 to 3 hours are required for the band to be visible and it remains readily detectable for 24 hours. The deep purple aldehyde-fuchsin complex is not bound to the enzyme or the gel and will diffuse throughout the gel and solution with time. Therefore, the samples must be observed and measured within 24 hours.

RESULTS

Preparation of Enzyme—The procedure of Ullrich et al. (8) was modified as described. Following ammonium sulfate fractionation, the 50 to 60% saturated precipitate was chromatographed on DEAE-cellulose. The elution profile is presented in Fig. 1. In all chromatographic experiments two activity peaks were observed. Multiple activity peaks on DEAE-cellulose have been reported before (22). Peak I is eluted at a conductivity between 3 and 4 millimhos measured at 25°C and the other between 4 and 5 millimhos measured at 25°C. Although the distribution of activity between Peak I and II varies with preparations, Peak I contains most of the activity. Peak I, the best defined material, was rechromatographed and used in most of the experimental work reported here. When rechromatographed, Peak I (Fig. 2) exhibits a single peak which is eluted under buffer conditions identical with the first DEAE-cellulose chromatogram.

A summary of the purification is presented in Table I, where the DEAE-cellulose-chromatographed material referred to is Peak I. The fold purification obtained ranged from 65 to 74 fold.

Disc Electrophoresis—The chromatographed material was further characterized with disc electrophoresis. Two staining procedures described under "Methods and Materials" were used: an Amido black for protein and a fuchsin staining reagent for the enzyme pyruvate decarboxylase. As indicated in Fig. 3, the protein stain reveals one predominant band with two trace bands. A duplicate electrophoresis sample was stained with the fuchsin staining reagent. The fuchsin-stained band, identified with pyruvate decarboxylase activity, coincided with the major protein band detected with Amido black.

Molecular weight was determined with the disc electrophoresis...
FIG. 3. Disc gel electrophoresis of pyruvate decarboxylase run at 7% acrylamide in the separating gel. For procedural details see text. Optical density units, 0.025, at 280 nm were applied to the gel. After electrophoresis protein bands were detected with Amido black. The arrow indicates the band which corresponds to enzymatic activity detected on a duplicate gel stained with the fuchsin staining reagent.

The technique described by Hedrick and Smith (23). The electrophoresis was carried out with the same buffer, gel systems, and operating conditions as described under "Methods and Materials" with one modification: the concentration of acrylamide in the separating gel was varied from 7 to 4% in 1% intervals. For each set of duplicate samples, one was stained with Amido black to detect protein and one was stained with the fuchsin staining reagent to identify the pyruvate decarboxylase. The distance that the material migrated in the varying gels was measured and a graphical analysis plotting \(100 \log(R_{2} \times 100)\) against percent gel was made. The slope of this line is proportional to the molecular weight (23). Results obtained in this study with ovalbumin, molecular weight 47,000, as a standard, agreed within 6% of the reported work cited (23). A slope of 8.06 ± 0.08 for the pyruvate decarboxylase was determined which corresponds with a molecular weight of 118,000 ± 2,600. These results suggest that enzyme activity is associated with a protein of a molecular weight 118,000 ± 2,600.

Effect of Buffer Species and pH on Enzyme Activity—The molecular weight of pyruvate decarboxylase determined in the above study was considerably lower than 175,000 molecular weight for active enzyme reported by Ulrich et al. (8). Reconsideration of the disc electrophoresis procedure recalled an observation made earlier in the course of studying this enzyme: a large loss of activity was noted when Tris-maleate buffer, pH 6.0, was used in the manometric assay. Since Tris-Cl was the buffer system used in preparation of the electrophoresis gels, it was important to consider the effect of Tris-Cl on enzyme activity. Tris-Cl at two concentrations was used for this purpose. The enzyme was added to the buffer and aliquots were removed at periodic intervals. The results are tabulated in Table II. In 0.50 M Tris-Cl, pH 6.7, at 4°F, 86% of the activity is lost in 1 hour, and in 0.025 M Tris-Cl, pH 6.7, at 4°F, 53% of the activity is lost during the same period. In contrast to these results, when the enzyme in 0.02 M

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. Incubation</td>
</tr>
<tr>
<td></td>
<td>medium: 0.50 M Tris-Cl, pH 6.7</td>
</tr>
<tr>
<td>min</td>
<td>%</td>
</tr>
<tr>
<td>0.5</td>
<td>72.3</td>
</tr>
<tr>
<td>10.0</td>
<td>52.3</td>
</tr>
<tr>
<td>30.0</td>
<td>23.8</td>
</tr>
<tr>
<td>60.0</td>
<td>13.5</td>
</tr>
</tbody>
</table>

The activity remaining was measured after 2 min at 4°F in 0.1 M potassium phosphate, pH 6.3, containing 25 μmoles of MgSO₄, and 1.5 μmoles of thiamine-PP in a total volume of 1.0 ml before initiating the reaction. • • •, potassium phosphate, 0.04 M, pH 7.5, previously incubated; ○ ○ ○, potassium phosphate, 0.04 M, pH 8.0, previously incubated; △ △ △, potassium phosphate, 0.04 M, pH 8.0, not first incubated; ■ ■ ■, Tris-Cl, 0.04 M, pH 7.5, previously incubated; □ □ □, Tris-Cl, 0.04 M, pH 8.0, previously incubated; ● ● ●, Tris-Cl, 0.04 M, pH 8.0, not first incubated.

2 \(R_{m}\) (relative mobility) = distance protein band travels/distance dye front travels.

Fig. 4. One-tenth milliliter of a protein solution, 13.16 mg per ml, prepared from the 50 to 60% ammonium sulfate precipitate was added to 0.9 ml of buffer. Final concentration was 1.32 mg per ml. The protein solutions were incubated at 4°F, aliquots were removed at selected time intervals as noted, and the activity was determined with the spectrophotometric procedure described. Two assays were performed. In one case the enzyme was assayed directly from the buffered solutions; in the second case the enzyme was first incubated for 2 min at 4°F in 0.1 M potassium phosphate, pH 6.3, containing 25 μmoles of MgSO₄, and 1.5 μmoles of thiamine-PP in a total volume of 1.0 ml before initiating the reaction. • • •, potassium phosphate, 0.04 M, pH 7.5, previously incubated; ○ ○ ○, potassium phosphate, 0.04 M, pH 8.0, previously incubated; △ △ △, potassium phosphate, 0.04 M, pH 8.0, not first incubated; ■ ■ ■, Tris-Cl, 0.04 M, pH 7.5, previously incubated; □ □ □, Tris-Cl, 0.04 M, pH 8.0, previously incubated; ● ● ●, Tris-Cl, 0.04 M, pH 8.0, not first incubated.

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Pyrurate Decarboxylase was chromatographed on Sephadex G-200 columns (90 × 1.5 cm) under varying pH and buffer conditions as indicated in the table, and the elution volume for each condition was determined. For procedural details see text.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Buffer</th>
<th>Elution volume of protein peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-cellulose-chromatographed enzyme</td>
<td>6.5</td>
<td>0.02 M potassium phosphate-0.1 M KCl</td>
<td>78</td>
</tr>
<tr>
<td>DEAE-cellulose-chromatographed enzyme</td>
<td>7.0</td>
<td>0.02 M potassium phosphate-0.1 M KCl</td>
<td>79</td>
</tr>
<tr>
<td>DEAE-cellulose-chromatographed enzyme</td>
<td>7.5</td>
<td>0.02 M potassium phosphate-0.1 M KCl</td>
<td>83</td>
</tr>
<tr>
<td>DEAE-cellulose-chromatographed enzyme</td>
<td>7.5</td>
<td>0.05 M Tris-Cl-0.1 M KCl</td>
<td>83</td>
</tr>
<tr>
<td>Reconstituted enzyme</td>
<td>8.0</td>
<td>0.02 M potassium phosphate-0.1 M KCl</td>
<td>76</td>
</tr>
<tr>
<td>Apoenzyme</td>
<td>6.5</td>
<td>0.02 M potassium phosphate-0.1 M KCl</td>
<td>87</td>
</tr>
</tbody>
</table>

As the pH is increased above 7 the enzyme is less stable. This was studied further comparing the stability of the enzyme in phosphate and Tris buffers. Enzyme stability was followed in phosphate buffer, pH 7.5 and 8.0, and Tris-Cl buffer, pH 7.5 and 8.0. The enzyme was added to the particular buffer system, and aliquots were removed at noted time intervals as indicated in Fig. 4. The activity was determined as soon as the aliquot was removed, before and after prior incubation with thiamine-PP and Mg^{2+} as described in the legend of Fig. 4. Preliminary incubation with thiamine-PP was performed as described by Schellenger and Hübner (7) to reconstitute the holoenzyme. Activity observed following preliminary incubation is comprised of two factors: (a) holoenzyme present and (b) activity obtained by reconstitution of holoenzyme. Direct assay without preliminary incubation with cofactors measures the amount of holoenzyme present. Results obtained by direct assay indicate that when the enzyme is exposed for 30 min to 0.01 M Tris-Cl buffer, pH 8.0, no holoenzyme is present. In contrast, when the enzyme is exposed for 10 min to phosphate buffer, pH 8.0, 25% of the activity is lost, suggesting that 75% of the holoenzyme is still present. When assayed following prior incubation with thiamine-PP and Mg^{2+}, enzyme exposed for 1 hour to 0.04 M potassium phosphate, pH 7.5, showed no loss of activity. If the enzyme is exposed to 0.04 M Tris-Cl buffer, pH 7.5, only 25% of the activity can be reconstituted. At pH 8.0 in 0.04 M potassium phosphate a 45% decrease in activity is observed in 100 min, but in 0.04 M Tris-Cl at pH 8.0 an 85% loss is observed. Two factors appear to be operative here: buffer species and pH. The enzyme is considerably more stable in potassium phosphate than in Tris-Cl. The decrease in activity at higher pH is consistent with previous reports (3-6) which have shown that at alkaline pH (8.0 and over) thiamine-PP is released from the enzyme and no activity is observed.

Chromatography on Sephadex G-200—The molecular weight of pyruvate decarboxylase determined by the disc electrophoresis technique of Hedrick and Smith (23) was in conflict with other reported studies (8). An additional determination by another technique, Sephadex G-200 chromatography, was undertaken. A Sephadex G-200 column, 90 × 1.5 cm, was prepared and calibrated with proteins in the calibration kit obtained from Pharmacia. The calibration curve is presented in Fig. 5. Chromatography of pyruvate decarboxylase was performed on this column under the following conditions: (a) 0.02 M potassium phosphate-0.1 M KCl, pH 6.5; (b) 0.02 M potassium phosphate-0.1 M KCl, pH 7.0; (c) 0.02 M potassium phosphate-0.1 M KCl, pH 7.5; (d) 0.05 M Tris-Cl-0.1 M KCl, pH 7.5; (e) 0.05 M Tris-Cl-0.1 M KCl, pH 8.0. The elution volume at each set of conditions is tabulated in Table III.

As the pH is raised the elution volume increases, suggesting dissociation and a change of the molecular weight of the protein unit. Superimposed upon the pH effect the buffer species also has an additional effect consistent with the stability studies. In Fig. 6 the elution profiles of the chromatogram performed in 0.02 M potassium phosphate-0.1 M KCl, pH 6.5, and the chromatogram performed in 0.05 M Tris-Cl-0.1 M KCl, pH 8.0, are presented. At pH 6.5 the elution volume is 78 ml and at pH 8.0 the elution volume is 93 ml. When the elution volume obtained
for these two conditions is plotted on the calibration curve one obtains a molecular weight of 209,000 at pH 6.5 and 108,000 at pH 8.0. At pH 7.5 the enzyme was chromatographed on the same Sephadex column in two buffer systems: 0.02 M potassium phosphate-0.1 M KCl and 0.05 M Tris-Cl-0.1 M KCl. When the potassium phosphate buffer was the equilibrating and eluting solution, the elution volume was 83 ml. When the Tris-Cl buffer was the equilibrating and eluting solution, the elution volume was identical with the results obtained at pH 8.0. As the pH is raised from 7 to 8 the protein dissociates into subunits of one-half the molecular weight of the original material. This dissociation appears to be enhanced in the presence of Tris-Cl compared with potassium phosphate.

Reconstitution of Enzyme Sephadex G-200 chromatography data indicate that pyruvate dehydrogenase dissociates into subunits of one-half the molecular weight when the pH is increased above 7.0. The information obtained from the enzyme stability studies indicates that activity is lost under the same conditions that protein dissociation occurs. Previous studies (3-6) based on activity determinations have indicated that reconstituted protein is identical with the original material. It seemed necessary at this juncture to determine what the state of aggregation of the reconstituted enzyme was.

Fifteen milligrams of protein obtained from DEAE-cellulose chromatography were incubated in 0.05 M Tris-Cl, pH 8.0, for 30 min at 4°C. An aliquot was removed for assay and the remaining solution was applied to a Sephadex G-25 column, 30 X 0.9 cm, equilibrated with 0.05 M Tris-Cl, pH 8.0. The fractions containing the protein separated from thiamine-PP were combined. To this protein sample cofactors were added so that the final concentrations were 1 mM MgCl$_2$ and 1 mM thiamine-PP. The protein solution was divided into two samples, A and B. Each was dialyzed separately according to the following order: (a) 0.05 M potassium phosphate, pH 7.5; (b) 0.05 M potassium phosphate, pH 7.0; (c) 0.05 M potassium phosphate, pH 6.5. Sample A was dialyzed against buffers containing 1 mM MgCl$_2$ and 0.1 mM thiamine-PP. Sample B was dialyzed against buffers containing 1 mM MgCl$_2$, 0.1 mM thiamine-PP, and 1 mM sodium pyruvate. The dialysates were changed at 40-min intervals. A final dialysis in 0.05 M potassium phosphate, pH 6.5, was carried out for each sample. The pH of each sample after dialysis was 6.5. Aliquots for each sample were removed for assay and 1.0 ml of the solution was applied to a Sephadex G-200 column equilibrated with 0.02 M potassium phosphate-0.1 M KCl, pH 6.5.

After incubation in 0.05 M Tris-Cl, pH 8.0, and prior to Sephadex G-25 chromatography the enzyme was assayed directly and after preliminary incubation with added cofactors. A trace of activity was noted when the enzyme was assayed directly; however, after preliminary incubation with the addition of cofactors 32% of the original activity was recovered. After separating the protein from the cofactors with Sephadex G-25 chromatography, the protein was assayed with and without prior incubation. Without previous incubation no activity was detected, while after preliminary incubation with thiamine-PP and Mg$^{2+}$ 58% of the activity was recovered. Aliquots of the apoenzyme left standing in 0.05 M Tris-Cl solutions were assayed after 2 and 24 hours. In both cases the enzyme was first incubated with cofactors. After 2-hour exposure to 0.05 M Tris-Cl, pH 8.0, at 4°C, 48% of the activity was recovered, while after 24-hour exposure to 0.05 M Tris-Cl, pH 8.0, at 4°C, 10% of the activity was recovered. The activity recovered decreased the longer the protein was exposed to 0.05 M Tris-Cl, pH 8.0. In sharp contrast, after the protein was dialyzed, gradually reducing the pH to 6.5 in the presence of the cofactors, over 100% of the original activity was obtained without preliminary incubation. The presence of sodium pyruvate in the dialysate did not have any effect.

The elution volume of the reconstituted enzyme chromatographed on the Sephadex G-200 column was 76 ml (Table III). These data show that the reconstituted enzyme has the same molecular weight as the original undissociated enzyme.

A parallel experiment was performed which was identical with the reconstitution experiment through the separation of the cofactors from the enzyme on Sephadex G-25 with 0.05 M Tris-Cl, pH 8.0. The enzymatic activity of the combined protein fractions was determined with and without preliminary incubation. Without previous incubation no activity was observed, and after preliminary incubation 29% of the activity was observed. The apoenzyme was dialyzed according to the same procedure and order of phosphate buffers described in the above experiment with one modification: the cofactors thiamine-PP and Mg$^{2+}$ were not present. After dialysis the protein was assayed again. The enzyme was completely inactive when assayed directly without prior incubation; after preliminary incubation with cofactors the same material was 87% active. The dialyzed protein was chromatographed on the Sephadex G-200 column equilibrated with 0.02 M potassium phosphate-0.1 M KCl, pH 6.5, the same conditions that were used for the chromatography of the original...
holoenzyme and the reconstituted enzyme. The elution volume of this material was 87 ml (Table III). Therefore, the apo-enzyme does not associate to form a stable dimer when thiamine-PP and Mg$^+$ are not present.

**DISCUSSION**

Preparation of pyruvate decarboxylase has consistently revealed two active protein peaks on DEAE-cellulose chromatography. As discussed previously, the ratio of the two peaks varies in different preparations from the same yeast source. Three possible explanations were considered: (a) isozymes, (b) different states of protein aggregation, and (c) modified enzyme produced during the preparative procedure. Since the ratio of the two activity peaks varies in different enzyme preparations from the same yeast source the presence of isozymes was excluded. A distribution of protein aggregates was excluded since on rechromatography of either Peak I or Peak II, one active peak was obtained which was eluted at the same position as on the initial chromatogram. The most plausible explanation is that these are artifacts resulting from some protein structural change which occurs during the preparative procedure. The presence of proteolytic enzymes in yeast autolysates has been reported by Juni and Heym (24, 25). Peptide bond cleavage catalyzed by such enzymes, thereby chemically altering the chemical structure, could produce multiple enzymatically active peaks. Further evaluation of the preparative procedure is required to answer this question. The first active peak, which contained most of the activity and had the highest specific activity representing a 65- to 74-fold purification, was the material used in this study.

Sephadex G-200 chromatography of the enzyme in 0.02 M potassium phosphate-0.1 M KCl, at pH 6.5 and 7.0, indicates that the molecular weight of pyruvate decarboxylase is 209,000. Chromatography on the same column in 0.05 M Tris-Cl-0.1 M KCl, pH 7.5 and 8.0, indicates that the molecular weight of the enzyme is 108,000 (Fig. 5). Therefore, it appears that in the phosphate buffer at pH 6.5 and 7.0 the protein is not dissociated; however, in the Tris-Cl buffer, pH 7.5 and 8.0, the protein is dissociated into subunits of one-half the molecular weight of the native material. In Tris-Cl buffer, pH 7.5, the enzyme is eluted at 93 ml (Table III); this indicates the same degree of dissociation as at pH 8.0. In potassium phosphate, pH 7.5, the enzyme is eluted at 83 ml, an intermediate position suggesting that the dissociation is not complete (Table III). These conflicting data raise a question as to the effect of the different buffer solutions. In the two solutions the pH and the ionic strength (phosphate buffer $\mu = 0.16$; Tris-Cl buffer $\mu = 0.14$) were the same. The only apparent difference was the buffer species. Evidence of some structural change affected by the pH is indicated by a shift in elution position observed with both buffers. However, in addition to the effect of pH, there appears to be some interaction between Tris and the enzyme that enhances protein dissociation.

The characteristics of the Sephadex G-200 chromatography in the two buffers can be correlated with the stability studies of the enzyme. The enzyme is stable for 18 to 24 hours in 0.05 M potassium phosphate, pH 6.8, at 4°, but in 0.50 M Tris-Cl, pH 6.7, at 4°, 86% of the activity is lost in 1 hour and in 0.025 M Tris-Cl, pH 6.7 at 4°, 55% of the activity is lost in 1 hour (Table II). As the pH is raised above 7.0 loss of activity is observed with both phosphate and Tris-Cl, but the rate of loss is always greater in the presence of Tris-Cl (Fig. 4). Consideration of this information with the Sephadex G-200 data suggests that above pH 7.0 loss of activity is associated with protein dissociation. It is possible that this also occurs below pH 7.0 when Tris is present. The loss of activity observed in the presence of Tris at pH 6.7 may be the result of this phenomenon. This aspect awaits further investigation.

Additional data which are consistent with protein dissociation are obtained from disc electrophoresis experiments. Previous reports (8, 9), as well as the stability study (Fig. 4) and chromatographic data reported (Table III), suggest that the associated protein in the 200,000 molecular weight range is enzymatically active and that the subunit is not. In contradiction to this, the molecular weight determined with the disc electrophoresis technique of Hedrick and Smith (23) associates the pyruvate decarboxylase activity with a protein of molecular weight 118,000 ± 2,000, thereby identifying enzymatic activity with a subunit of approximately one-half the molecular weight. These disparate facts can be resolved by consideration of the electrophoretic conditions and the correlation between the enzymatic activity and protein dissociation as a function of pH and buffer species (Fig. 4 and Table II). In the disc electrophoresis procedure the enzyme was exposed to 0.15 M Tris-Cl, pH 6.7, in the sample and stacking gel and 0.19 M Tris-Cl, pH 8.3, in the separating gel. The conditions in the separating gel favor dissociation. In the process of detecting the pyruvate decarboxylase activity with the fuchsin staining reagent the gel is placed into a medium containing all of the required cofactors at pH 6.0. As the fuchsin staining reagent permeates the gel, it replaces the electrophoresis buffer, the pH decreases to 6.0, and thiamine-PP and Mg$^+$ are present. These are conditions that would favor reassociation of the holoenzyme. Therefore, it would appear that the enzyme is dissociated during migration in the separating gel and is reconstituted to active holoenzyme during incubation with the fuchsin staining reagent. Under the conditions of the disc electrophoresis the molecular weight of the migrating unit is not the same as the catalytically active unit in this particular case. Thus the data from the disc electrophoresis molecular weight determination which appear contradictory can be understood in a context which is consistent with protein dissociation.

Coincident with these studies, chromatography on Sephadex G-25 confirmed previous reports (5, 6) that thiamine-PP was released and could be separated from the protein component in 0.05 M Tris-Cl, pH 8.0, and was not released in the presence of 0.02 M potassium phosphate, pH 6.5. Thus the evidence accrued strongly suggests that the protein dissociates into subunits under the same conditions that the cofactors are released and can be separated from the protein.

The molecular weight of the holoenzyme and the subunit determined with Sephadex G-200 are greater than the values of each previously reported (8, 9). This may be attributed to the different techniques and conditions used. Andrews (26) indicates a precision of ±10% when the elution volume is measured to the nearest milliliter, and Winzor (27) suggests that ±20% is not unreasonable for molecular weight determined by gel filtration. The results reported here have been consistent and reproducible in the Sephadex system used during the course of this investigation. The critical factor is that the ratio of the molecular weight at pH 6.5 to that at pH 8.0 is 1.9, indicating that the protein dissociates into two subunits of one-half the molecular
weight of the holoenzyme. These data are consistent with the subunit determination of denatured enzyme reported by Ullrich and Kemple (9).

Reconstitution of holoenzyme as determined by activity measurements depends on the length of time the subunit is exposed to 0.05 M Tris-Cl, pH 8.0. The activity recovered decreases the longer the enzyme is left at pH 8.0. Therefore, it appears that the subunit is labile and is irreversibly denatured at these conditions. The most favorable conditions for reconstitution appear to be a gradual decrease in pH from 8.0 to 6.5 in the presence of the cofactors thiamine-PP and Mg²⁺. Previous investigators (3-6) have indicated that reconstituted enzyme was identical with the original material. This conclusion was based on activity determinations. In conjunction with this it was of interest to determine the chromatographic behavior of reconstituted enzyme on Sephadex G-200 chromatography. At pH 6.5 the elution volume for the reconstituted material was 76 ml, which is comparable to a molecular weight of 225,000 in the system used. This is within experimental error of the procedure and indicates that the molecular weight of the reconstituted material is identical with the original holoenzyme.

Formation of holoenzyme in the presence of Mg²⁺ and thiamine-PP with the same molecular weight as the original enzyme prompted an investigation of the association properties of the apoenzyme in the absence of cofactors. As shown in Table III, when the apoenzyme prepared at pH 8.0 is carried through the same procedure for the formation of the holoenzyme without thiamine-PP and Mg²⁺, the elution character of this material is significantly different. The apoenzyme at pH 6.5 is eluted at 87 ml, an intermediate position in the direction of the monomer unit. It is of interest to compare the three Sephadex G-200 chromatography experiments at pH 6.5: (a) the original enzyme, (b) the reconstituted holoenzyme, and (c) the apoenzyme. In the first two cases the elution profiles were symmetrical. The elution profile of the apoenzyme at pH 6.5 was not as symmetrical. An elution position intermediate between the monomer-dimer positions could be a reflection of a rapid concentration dependent equilibrium of a monomer-dimer system. In this instance, the elution position is in the direction of the monomer unit. The result therefore indicates that the protein which dissociates at pH 8.0 into 2 units of one-half the original molecular weight forms a stable dimer in the presence of thiamine-PP and Mg²⁺ when the pH is decreased to 6.5. There was no evidence of monomer within the limit of detection of the chromatographic procedure used. When the apoenzyme is put through the reconstitution procedure described in the absence of thiamine-PP and Mg²⁺ there is evidence for some association as indicated by the elution position, and in this case the monomer is favored. These results indicate that thiamine-PP, in addition to its catalytic role, also functions in the formation of a stable dimer which is equivalent to the active holoenzyme. For many years investigators of this enzyme have remarked on the unusual requirement for excess TI-P to reconstitute holoenzyme (3-6). A protein association step which requires the presence of thiamine-PP and Mg²⁺ in order to form an active dimer identifies a locus in the reconstitution process which may be related to this question. At this time there is not sufficient evidence to describe this process in greater detail. Many questions remain: e.g. (a) are thiamine-PP and Mg²⁺ released before, after, or in a concerted step with protein dissociation; (b) are the two subunits identical (Ullrich and Kemple (9) have suggested that they are); and (c) can these subunits be dissociated further?

It is of interest to note that the pyruvate decarboxylase component of the pyruvate dehydrogenase complex of *Escherichia coli* (28) is also dissociated into subunits at alkaline pH. The mechanism of the reactions catalyzed by the α-keto dehydrogenases are similar. Thiamine-P₃ and Mg²⁺ are obligatory in every case. In particular the formation of the thiamine-PP-pyruvate aduct followed by the elimination of CO₂ producing hydroxymethyl thiamine-PP appears to be the initial step common to all of the enzymes in this category. It will be of interest to follow the similarities and potential subtle variations in the protein structure and molecular organization of the pyruvate dehydrogenases which range from independent enzymes as the cytoplasmic yeast enzyme reported here to components of macro-molecular complexes such as the pyruvate dehydrogenase (28).

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Pyruvate Decarboxylase: I. PROTEIN DISSOCIATION INTO SUBUNITS UNDER CONDITIONS IN WHICH THIAMINE PYROPHOSPHATE IS RELEASED
Anne D. Gounaris, Iris Turkenkopf, Sharon Buckwald and Anne Young


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