Some Properties of the Pyruvate Carboxylase from *Pseudomonas fluorescens*

By SILVIA R. MILRAD DE FORCHETTI AND J. J. CAZZULO

Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 531, Rosario, Argentina

(Received 28 May 1975; revised 14 September 1975)

**SUMMARY**

The pyruvate carboxylase of *Pseudomonas fluorescens* was purified 160-fold from cells grown on glucose at 20 °C. The activity of this purified enzyme was not affected by acetyl-coenzyme A or L-aspartate, but was strongly inhibited by ADP, which was competitive towards ATP. Pyruvate gave a broken double reciprocal plot, from which two apparent \( K_m \) values could be determined, namely 0.08 and 0.21 mM, from the lower and the higher concentration ranges, respectively. The apparent \( K_m \) for HCO\(_3^-\) at pH 6.9, in the presence of the manganese ATP ion (MnATP\(^{2-}\)), was 3.1 mM. The enzyme reaction had an optimum pH value of 7.1 or 9.0, depending on the use of MnATP\(^{2-}\) or MgATP\(^{2-}\), respectively, as substrate. Free Mg\(^{2+}\) was an activator at pH values below 9.0. The enzyme was strongly activated by monovalent cations; NH\(_4^+\) and K\(^+\) were the better activators, with apparent \( K_a \) values of 0.7 and 1.6 mM, respectively. Partially purified enzymes from cells grown on glucose at 1 or 20 °C had the same properties, including the thermal stability. In both cases 50% of the enzyme activity was lost after pre-incubation for 10 min at 46 °C. The molecular weight was estimated to be about 300000 daltons by gel filtration on Sephadex G-200. The regulatory properties and molecular weight are thus similar to those determined for the pyruvate carboxylases from *Pseudomonas citronellolis* and *Azotobacter vinelandii*.

**INTRODUCTION**

*Pseudomonas fluorescens* contains two enzymes able to synthesize oxaloacetate by carboxylation of a three-carbon precursor: pyruvate carboxylase (EC 6.4.1.1) and phosphoenolpyruvate carboxylase (EC 4.1.1.31) (Higa, Milrad de Forchetti & Cazzulo, 1976). The preliminary study of the pyruvate carboxylase activity in cell-free extracts showed it to be independent of acetyl-coenzyme A (acetyl-CoA) and insensitive to L-aspartate (Higa et al., 1976), as previously demonstrated for the same enzyme in *Ps. citronellolis* (Scrutton & Young, 1972) and *Azotobacter vinelandii* (Scrutton & Taylor, 1974). This paper describes the preparation of highly purified pyruvate carboxylase from glucose-grown *Ps. fluorescens* and some of its properties, particularly those related to the effects of adenine nucleotides and divalent and monovalent cations on the enzyme activity.

**METHODS**

Preparation of pyruvate carboxylase. The enzyme was purified by a method similar to that described by Cazzulo, Sundaram & Kornberg (1970) for the purification of the pyruvate carboxylase from *Bacillus stearothermophilus*. *Pseudomonas fluorescens*, strain R12, was...
grown at 20 °C on glucose as sole carbon source. A crude extract of the cells was prepared and treated with protamine sulphate as described by Cazzulo et al. (1970). Solid ammonium sulphate to 75% saturation was added to the supernatant at 0 °C. The precipitate was removed by centrifugation for 30 min at 37000 g and 0 °C, and extracted with ammonium sulphate solutions of decreasing concentration in water containing 1 mM-EDTA and adjusted to pH 7.0, essentially as described by Cazzulo et al. (1970) except that the ammonium sulphate concentrations (w/v) and the amounts of solutions added were as follows: 28% (6 ml), 23.6% (two extractions, 3 ml each), 19% (2 ml), 18% (2 ml), and 15.3% (1 ml). The bulk of the pyruvate carboxylase activity was recovered in the 18% and 19% ammonium sulphate solutions, which were pooled, precipitated by a solution of ammonium sulphate of 80% saturation and centrifuged. The precipitate was dissolved in 0.8 ml of 0.05 M-tris-HCl buffer pH 7.6 containing 1 mM-EDTA and 0.4 M-KCl, and applied to the top of a column of Sephadex G-200 (35.2 x 1.2 cm), which was eluted with the same buffer at a rate of 6.3 ml/h, at 1 °C. Fractions (1 ml) were collected and assayed for protein and pyruvate carboxylase activity. The most active fractions were pooled, dialysed overnight against 100 vol. of 10 mM-potassium phosphate buffer pH 7.0 containing 0.1 mM-EDTA and 0.4 M-KCl, and applied to the top of a hydroxylapatite column (1.6 x 1.0 cm) equilibrated with the same buffer solution. The column was washed with the same buffer and eluted with buffer solutions of similar composition, containing 90 and 110 mM-potassium phosphate, respectively, at a rate of 4 ml/h. The effluent was collected in 2 ml fractions. The most active fractions eluted by the 110 mM-potassium phosphate buffer were combined.

The purity of the enzyme preparations was tested by polyacrylamide gel electrophoresis, performed essentially as described by Cazzulo et al. (1971), except that in some experiments 5 M-urea was added both to the solutions employed for the preparation of the gels and to the reservoir buffer.

Assay methods. Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard (steps 1 to 3 of the purification procedure) or by the spectrophotometric method of Warburg & Christian (1941) (steps 4 and 5).

Pyruvate carboxylase was assayed with a Unicam SP 1800 B recording spectrophotometer (Pye Unicam Ltd, Cambridge) by measuring the decrease in extinction at 340 nm concomitant with the oxidation of NADH at 30 °C. Unless stated otherwise, the reaction mixtures contained in a final volume of 1 ml (μmol): tris–HCl buffer pH 8.5, 100; sodium pyruvate, 5; ATP, 2; MgCl₂, 5; KCl, 10; NaHCO₃, 15; NADH, 0.15; and 0.5 i.u. malate dehydrogenase (EC. 1.1.1.37); and enzyme as stated. The reaction was started by the addition of ATP and measured after 1 min. The results were corrected for the ATP-independent oxidation of NADH observed with enzyme preparations from steps 1 and 2, due to the presence of lactate dehydrogenase (EC. 1.1.1.27). One unit of enzyme activity is defined as that amount which catalyses the synthesis of 1 μmol oxaloacetate (determined by the oxidation of 1 μmol NADH)/min under the standard assay conditions described; the specific activity is expressed as units of enzyme/mg protein. When the lactate dehydrogenase activity of the samples was high, as in the experiment for the determination of the molecular weight, pyruvate carboxylase was assayed in a reaction mixture similar to that described above, with 1 i.u. citrate synthase, 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB] and 0.15 mM-acetyl-CoA substituted for malate dehydrogenase and NADH. The reaction was followed as the increase in extinction at 412 nm, due to the reaction of DTNB with the CoA liberated in the coupled reaction. The approximate molecular weight of the pyruvate carboxylase was determined by gel filtration through Sephadex G-200, according to Andrews (1965). The experimental conditions were similar to those used for the gel
Pyruvate carboxylase of *Ps. fluorescens*

filtration step of the purification method. The proteins used as markers were malate dehydrogenase (0.05 mg; molecular weight 55,000 to 70,000 daltons), citrate synthase (0.01 mg; 140,000 to 150,000 daltons), catalase (0.4 mg; 195,000 daltons), and pyruvate kinase (0.03 mg; 230,000 to 250,000 daltons). The corresponding elution volumes were 26.0, 24.8, 21.6, 19.5 and 18.1 ml, respectively. The elution volume of pyruvate carboxylase (0.5 mg, step 4 of the purification method) was 16.5 ml. The void volume of the column, determined with Blue Dextran 2000, was 13.1 ml. The molecular weight given for catalase is the apparent molecular weight calculated from gel filtration experiments (Andrews, 1965). Malate dehydrogenase, lactate dehydrogenase and catalase (EC. 1.11.1.6) were assayed as described by Andrews (1965), citrate synthase (EC. 4.1.3.7) as described by Srere (1969), and pyruvate kinase (EC. 2.7.1.40) as described by Bücher & Pfleiderer (1955).

**Chemicals.** Protamine sulphate was obtained from Sigma, lactate dehydrogenase, catalase and citrate synthase from Boehringer, Mannheim, Germany, and Sephadex G-200 and Blue Dextran 2000 from Pharmacia. Hydroxylapatite was prepared according to Tiselius, Hjertén & Levin (1956). All other chemicals were as previously described (Higa *et al.*, 1976).

**RESULTS AND DISCUSSION**

The purification method described, which is summarized in Table 1, resulted in a 160-fold purification of pyruvate carboxylase. The low yield was due in part to the fact that the fractions of lower specific activity in steps 3 to 5 were discarded, and to the increased instability of the purified enzyme. The purified preparation from step 5, which was free of phosphoenolpyruvate carboxylase activity, showed two main protein bands when subjected to polyacrylamide gel electrophoresis, but apparently only one protein band when the gel electrophoresis was performed in the presence of 5 mM-urea, suggesting that the preparation was essentially homogeneous but consisted of different aggregation forms of the enzyme. If one of these enzyme forms was inactive, it could explain the lower specific activity of the *Ps. fluorescens* enzyme in comparison with the values reported for pyruvate carboxylases purified from other sources (Scrutton & Taylor, 1974).

The highly purified enzyme, assayed spectrophotometrically, showed the same requirements for activity as those demonstrated by the radiochemical assay for the crude enzyme preparation (Higa *et al.*, 1976). Its activity was not affected by acetyl-CoA concentrations up to 0.15 mM or by L-aspartate concentrations up to 10 mM. Avidin was able to abolish the enzyme activity, and this inhibition was prevented by preincubation of the avidin with D-biotin. The requirement for HCO₃⁻ was difficult to demonstrate at pH 8.5 because of the CO₂ dissolved in the buffer solution; it could be demonstrated, however, at pH 6.9, with Mn²⁺ as the divalent cation activator (see below); under such conditions, the apparent *Kₘ* for NaHCO₃ was 3.1 mM. When the relationship of enzyme activity to the concentration of sodium pyruvate was studied, the double reciprocal plot (Lineweaver & Burk, 1934) consisted of two linear intersecting parts (Fig. 1); the one corresponding to the lower pyruvate concentration range yielded an apparent *Kₘ* of 0.08 mM, and the other a *Kₘ* of 0.21 mM. Similar broken double reciprocal plots with pyruvate have been observed for other pyruvate carboxylases (Cazzulo *et al.*, 1970; Scrutton & Taylor, 1974).

Figure 2 shows the enzyme activity as a function of the pH of the reaction mixture, determined with ATP and MgCl₂ at equimolar concentrations, or with a twofold excess of MgCl₂, or with equimolar concentrations of ATP and MnCl₂. As previously demonstrated for the pyruvate carboxylases from baker’s yeast (Cazzulo & Stoppani, 1969a), *B. stearo-
Pyruvate carboxylase was purified from 20.7 g cells grown on glucose at 20 °C, and assayed, as described in Methods.

Table 1. Purification of the pyruvate carboxylase from *Pseudomonas fluorescens*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>49</td>
<td>1145</td>
<td>65.1</td>
<td>0.06</td>
<td>× 1</td>
<td>100</td>
</tr>
<tr>
<td>2. Protamine sulphate supernatant</td>
<td>56</td>
<td>653</td>
<td>45.9</td>
<td>0.07</td>
<td>× 1.2</td>
<td>70</td>
</tr>
<tr>
<td>3. Ammonium sulphate fractionation</td>
<td>8</td>
<td>82.8</td>
<td>33.5</td>
<td>0.40</td>
<td>× 6.6</td>
<td>51</td>
</tr>
<tr>
<td>4. Sephadex G-200 column fractionation</td>
<td>5.9</td>
<td>20.9</td>
<td>8.1</td>
<td>3.87</td>
<td>× 64.5</td>
<td>12.4</td>
</tr>
<tr>
<td>5. Hydroxylapatite column fractionation</td>
<td>10.1</td>
<td>0.18</td>
<td>1.7</td>
<td>9.34</td>
<td>× 157</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of the concentration of pyruvate on the activity of pyruvate carboxylase from *Ps. fluorescens*. The reaction velocity was measured using 11 μg enzyme/assay. The experimental conditions were as described, except for the concentration of pyruvate, which was varied as shown.

Fig. 2. Effect of pH on the activity of pyruvate carboxylase from *Ps. fluorescens*. Activity was assayed using 11 μg enzyme/assay in 0.1 M-tris-acetate buffer, containing 1 mM-ATP and 1 mM-MgCl₂ (○), 1 mM-ATP and 1 mM-MnCl₂ (△), or 1 mM-ATP and 2 mM-MgCl₂ (●).

Thermophilus* (Cazzulo et al., 1970) and Verticillium albo-atrum* (Hartman & Keen, 1974), the optimum pH value depended on the nature of the cation, being about 7.1 with MnCl₂ and about 9.0 with MgCl₂, at equimolar concentrations of ATP and divalent cations. Excess MgCl₂ activated the enzyme at pH values below 9.0. MnCl₂ was better than MgCl₂ as an activator at pH 7.0, and the opposite was true at pH 9.0. Figure 3 shows that the plot of enzyme activity as a function of the concentration of ATP was sigmoideal when the concentrations of MgCl₂ was kept equal to that of the nucleotide. When 3 mM-MgCl₂ was present in excess over the magnesium ATP ion (MgATP₂⁻) concentration the curve was converted to a hyperbola. Similar results have been reported for other pyruvate carboxylases and attributed to the activation of the enzyme by free Mg²⁺ (Keech & Barritt, 1967; Cazzulo et al., 1970). However, the metal in excess may also act by converting the remaining free ATP⁺⁻, which has been demonstrated as an inhibitor of some of these enzymes, into the complex ion MgATP₂⁻, the true substrate of the reaction (Cazzulo & Stoppani, 1969a).

ADP was a strong inhibitor of the pyruvate carboxylase from *Ps. fluorescens* (Fig. 4);
Pyruvate carboxylase of *Ps. fluorescens*

0.5
1.0
1.5
2.0

ATP concn (mM)

Fig. 3

0
3
1
2

ADP or AMP concn (mM)

Fig. 4

This effect could not be due to the formation of a complex with the free Mg$^{2+}$ required as an activator, as Mg$^{2+}$ was in excess over the total concentration of nucleotide present in the reaction mixture. Under similar conditions and concentration AMP did not inhibit the enzyme (Fig. 4). The inhibition by ADP was competitive with ATP, as demonstrated by the double reciprocal plots shown in Fig. 5. The apparent $K_m$ for ATP (with 3 mM-MgCl$_2$ in excess over the concentration required to form the MgATP$^{2-}$ complex) increased from 0.09 mM in the absence of ADP to 1.43 mM in the presence of 1.5 mM-ADP. Similar inhibitory effects of ADP have previously been reported for the pyruvate carboxylases from baker’s yeast (Cazzulo & Stoppani, 1969b) and *B. stearothermophilus* (Cazzulo et al., 1970).

As shown for other pyruvate carboxylases (Scrutton & Young, 1972; Scrutton & Taylor, 1974) the enzyme from *Ps. fluorescens* was strongly activated by monovalent cations (Fig. 6). Absolute dependence on added monovalent cation could not be demonstrated, probably because of the presence of traces of cations in the enzyme preparation and the reagents. NH$_4$Cl and KCl were considerably better activators than LiCl and NaCl, both in terms of apparent $V_{max}$ attained and of apparent $K_a$ values; the latter values, calculated from double reciprocal plots considering only the increase in enzyme activity due to the added cations in Fig. 6, were 0.7, 1.6, 23 and 26 mM, respectively. Tris–HCl seemed to cause a slight inhibition.

The molecular weight of the pyruvate carboxylase from *Ps. fluorescens*, as determined by gel filtration through Sephadex G-200 (Andrews, 1965), was about 300000, similar to the molecular weights of the enzymes from *Ps. citronellolis* (Scrutton & Young, 1972) and *A. vinelandii* (Scrutton & Taylor, 1974). This fact, together with the lack of effect of both acetyl-CoA and L-aspartate, suggests that the *Ps. fluorescens* enzyme also belongs to type V in the classification of Scrutton & Young (1972).

*Pseudomonas fluorescens* can be considered as a facultative psychrophile, being able to grow at 1°C and unable to grow at 41°C, with an optimum temperature for growth of about 25°C (Doudoroff & Palleroni, 1974). It was considered of interest to study the thermal stability of the enzyme, as well as the properties of the enzyme produced at different
Fig. 5. Inhibition of pyruvate carboxylase from *Ps. fluorescens* by ADP. The experimental conditions were as described in the legend to Fig. 3, with the concentration of ATP varied as shown and the MgCl₂ concentration 3 mM in excess of that of ATP. ○, without ADP; ●, with 1.25 mM-ADP.

Fig. 6. Effect of monovalent cations on the activity of pyruvate carboxylase from *Ps. fluorescens*. The reaction mixtures contained in a final volume of 1 ml: tris-HCl buffer pH 8.5, 25 μmol; sodium pyruvate, 2 μmol; disodium ATP, 1 μmol; MgCl₂, 5 μmol; NaHCO₃, 5 μmol; NADH, 0.15 μmol; malate dehydrogenase, 0.5 i.u.; enzyme, 11 μg; and NH₄Cl (○), KCl (●), LiCl (△), NaCl (▲) or tris-HCl (□) as indicated. Pyruvate carboxylase and malate dehydrogenase were dialysed immediately before use against 50 mM-tris-HCl buffer pH 7.6 containing 1 mM-EDTA, at 1 °C for 5 h, with three changes of buffer.

Temperatures. The thermal stability of the highly purified enzyme obtained after step 5 of the purification method was studied by heating for 10 min, at different temperatures ranging from 0 to 50 °C, a preparation (19 μg of protein/ml) previously dialysed against 100 vol. of tris-HCl buffer pH 7.6 containing 1 mM-EDTA. Under such conditions the enzyme was stable up to 40 °C and lost 50% of the initial activity at 46 °C. The thermal stability of enzyme purified to step 4 of the purification method from cells grown on glucose at 1 °C and showing identical regulatory properties and molecular weight, was similar. The thermal stability of the pyruvate carboxylase from *Ps. fluorescens* was greater than that of the enzyme present in crude extracts of *Bacillus licheniformis* (Sundaram, Cazzulo & Kornberg, 1969) but lower than that of the purified pyruvate carboxylase from *B. stearothermophilus* (Cazzulo et al., 1970). In the case of phosphoenolpyruvate carboxylase, on the other hand, the enzyme from a marine psychrophile, with temperature limits for growth similar to those for *Ps. fluorescens*, was considerably less thermostable than the enzyme from mesophiles (Vidal & Cazzulo, 1972).

This research was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina and the Consejo de Investigaciones de la Universidad Nacional de Rosario, Argentina. J.J.C. is a member of the Carrera del Investigador Científico, and S.R.M.F. holds a scholarship from the former institution.
REFERENCES


