Pyruvate Decarboxylase of Zymomonas mobilis: Isolation, Properties, and Genetic Expression in Escherichia coli

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Pyruvate decarboxylase (EC 4.1.1.1) from Zymomonas mobilis was purified to homogeneity by using dye-ligand and ion-exchange chromatography. Antibodies produced against the enzyme and the aminoterminal sequence obtained for the pure enzyme were used to select and confirm the identity of a genomic clone encoding the enzyme selected from a genomic library of Z. mobilis DNA cloned into pUC9. The genomic fragment encoding the enzyme expressed high levels of pyruvate decarboxylase in Escherichia coli. Possible RNA polymerase and ribosome-binding sites have been identified in the 5'-untranslated region of the pyruvate decarboxylase gene.

Rapid and exclusive production of ethanol as the end product of sugar fermentation by the bacterium Zymomonas mobilis is due to the presence of pyruvate decarboxylase, an enzyme rarely found in bacteria, more commonly occurring in fungi and plants. The activity of the enzyme in Z. mobilis, at around 600 μmol min⁻¹ g⁻¹ (wet weight)⁻¹ (1), is higher than in any other source; even yeasts do not have such high levels in their cytoplasm (32). The enzyme constitutes over 5% of the readily soluble protein in Z. mobilis grown under normal conditions with glucose as substrate, at 30 to 32°C, pH 5 to 6. The virtual absence of proteolytic activity in extracts of Z. mobilis makes this an ideal source for purifying the enzyme; yeast pyruvate decarboxylase is notoriously susceptible to proteolytic degradation during isolation (8).

Hoppenr and Doelle (9) reported an isolation procedure for the Z. mobilis enzyme. Although the specific activity was quite high, the yield was very low. The minimum amount of activity necessary to sustain the fermentative flux at 30°C is 10 times higher than the amount they extracted. Other purification procedures have also been evolved (L. O. Ingram, S. Bringer-Meyer, personal communications). The present paper presents an isolation procedure which results in greatly improved yields, up to 4 mg of purified enzyme from every gram (wet weight) of cells.

Since the level of enzyme in Z. mobilis is so high, the promoter and ribosome-binding sites of the pyruvate decarboxylase gene are likely to be near ideal for the Z. mobilis protein expression system. With a view to investigating the structural features of the transcriptional control region of this gene, we have made a genomic library of Z. mobilis DNA, selecting the 2- to 6-kilobase fragments from a partial Sau3A digest, recombined into plasmid pUC9 in Escherichia coli, and have selected clones expressing active pyruvate decarboxylase. After N-terminal protein sequencing, a DNA probe was constructed that enabled nucleotide sequencing covering a region 200 bases upstream of the structural gene; this contains possible RNA polymerase-binding sites and a putative purine-rich ribosome-binding site spaced 6 bases upstream of the initiation codon.

Reagents and enzymes. Thiamine pyrophosphate, sodium pyruvate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl- β-D-thiogalactopyranoside (IPTG) were obtained from Sigma Chemical Co. The deoxydideoxyxynucleoside triphosphates were from Boehringer-Mannheim. All restriction endonucleases were obtained from New England BioLabs or Bethesda Research Laboratories. Nitrocellulose filters (BA85) were from Schleicher & Schuell. All other chemicals were analytical grade.

Z. mobilis strain ZM6 (ATCC 29191) was obtained from P. L. Rogers, University of New South Wales, Sydney, Australia. It was grown in conditions described elsewhere (26, 33), using 15 to 18% glucose as carbon substrate, harvested by centrifugation, washed, and stored as a wet paste.

E. coli strain JM101 was used as the host organism for plasmid pUC9 (15).

Extraction of cells. For protein isolation, Z. mobilis cells were lysed with Nonidet P-40 and lysozyme as described previously (26), using a pH 6.5 buffer. Enzyme activity was lost if the extract was exposed to a pH of ~7.0. Recombinant E. coli cells were extracted by a similar procedure, with the addition of vigorous vortexing in the presence of 50- to 150-μm glass beads (Sigma) (32).

Dye-ligand and ion-exchange chromatography. Dye-ligand adsorbents, prepared as described previously (18), were screened for binding of pyruvate decarboxylase. The buffer used was 10 mM KOH, adjusted to pH 6.0 with solid morpholinooethanesulfonate (MES) and containing 30 mM NaCl and 2 mM MgCl₂. At pH 5.5, this same buffer was adjusted with acetic acid. All buffers contained 0.1 mM thiamine pyrophosphate. In the isolations described, the dye-ligand adsorbents used were Green H-E4BD Sepharose CL-4B (dye content, 1.3 mg g⁻¹ wet weight) and Yellow H-E4R Sepharose CL-4B (dye content, 5.4 mg g⁻¹ wet weight). These Procion dyes were obtained from ICI Australia. The extract (400 ml) containing 5,000 mg of protein was run on a Green H-E4BD Sepharose CL-4B column (16 cm² by 12 cm) at pH 6.0 in MES buffer and washed with buffer exactly as described in the purification of 6-phosphogluconolactonase (24). The nonadsorbed material containing pyruvate decarboxylase was collected and its pH was lowered to 5.5.
with 1 M acetic acid. This was run on a Yellow H-E4R Sepharose CL-4B column (16 cm² by 8 cm), and nonadsorbed material was washed out with pH 5.5 buffer. Proteins including pyruvate decarboxylase were eluted by a change to pH 6.0 buffer containing 0.5 M NaCl. The proteins were precipitated from solution by dissolving 50 g of ammonium sulfate per 100 ml, and the precipitate was collected by centrifugation. The isolation could be interrupted for several days at this point.

The pH 7.0 buffer for ion-exchange chromatography was made by adjusting 10 mM HCl to pH 7.0 with imidazole and adding MgCl₂ to 2 mM. The precipitated proteins were dissolved in 40 ml of pH 7.0 buffer and desalted on a 500-ml column of Sephadex G-25 into the pH 7.0 buffer. A column of DEAE-Trisacryl (LKB Produkter) or DEAE-Sephasel (Pharmacia) (8 cm² by 20 cm) was equilibrated with the buffer and the desalted sample was applied. The enzyme was eluted with the aid of a gradient formed with 500 ml of 40 mM MgCl₂ in buffer, mixing into 500 ml of the buffer without MgCl₂ in identical beakers. Pyruvate decarboxylase was eluted about halfway through the gradient, clear of other contaminants. The activity of pyruvate dehydrogenase was monitored by ultraviolet spectroscopy at 250 nm, and solid ammonium sulfate was added until a turbidity developed. A sheen of microcrystals formed after a few days or weeks. The enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 1.5-mm vertical slab gels (12).

**Enzyme assay and protein measurement.** Pyruvate decarboxylase activity was measured in potassium-MES buffer, pH 6.5 (ionic strength, 0.05), containing 5 mM MgCl₂ and 0.1 M thiamine pyrophosphate. The buffer also contained 0.1 mg of bovine serum albumin ml⁻¹, 0.15 mM NADH, and 10 µM of yeast alcohol dehydrogenase ml⁻¹ (25). Pyruvate was added to 5 mM, and the rate of NADH oxidation was measured. Activity is expressed in micromoles per minute at 25°C. Protein was measured by the dye-binding method (27), and the pure enzyme was quantitated by measuring A 205/280 (23).

**Amino acid sequencing.** A 40-µg (0.67-nmol subunits) portion of pure pyruvate decarboxylase was subjected to NH₃ terminal sequence analysis by automated Edman degradation, using an Applied Biosystems gas-liquid-phase sequencer (7), and the phenylthiohydantoin amino acids were analyzed by reversed-phase high-pressure liquid chromatography with a Zorbax ODS column (34).

**Antibody production.** Antibodies against pyruvate decarboxylase were raised in rabbits by injecting an emulsion containing 500 µg of enzyme in complete Freund adjuvant followed by further monthly injections of 250 µg of enzyme in incomplete adjuvant. The animal was bled 6 days after the injections, and the antibodies were purified from the serum on an affinity column. Approximately 10 mg of pyruvate decarboxylase was coupled to 2 ml of tTesyl-activated Sepharose (19), and 1 to 2 ml of serum was passed through. Anti-pyruvate decarboxylase antibodies were eluted with 0.05 M glycine-HCl (pH 2.5)–3 M NH₄SCN with 10% dioxan, and the eluate was adjusted to pH 7.5 with 3 M Tris and precipitated by adding solid ammonium sulfate to 40% saturation.

**Construction and screening of library.** Z. mobilis chromosomal DNA was isolated by the procedure of Saito and Metcalf (21). A DNA fragment with 50 units of proteinase K ml⁻¹ was included prior to extraction with phenol.

The high-molecular-weight DNA was digested with Sau3A under conditions that maximized the number of fragments of 2- to 6-kilobase length and electrophoresed in submarine agarose gels (13). For preparative work, Pharmacia NA agarose was used, with electroelution of the required bands into dialysis tubing (13). The partially digested Sau3A fragments were ligated into the phosphatased BamHI site of plasmid pUC9, using DNA ligase, and used to transform _E. coli_ strain JM101 (6). Colonies were grown on nutrient agar containing ampicillin (100 µg ml⁻¹), X-Gal (160 µg ml⁻¹), and IPTG (190 µg ml⁻¹) to distinguish recombinants. Recombinants were harvested in 2 x 10 ml yeast extract, 5 g of NaCl liter⁻¹, and the library was stored at −20 or −70°C in 50% glycerol.

Recombinants were plated without IPTG induction on nitrocellulose filter overlays on nutrient agar containing ampicillin (100 µg ml⁻¹). The colonies on replica filters were lysed in situ and probed with affinity-purified antibodies and 1 µCi of [153I]labeled protein A (10).

 Autoradiography was performed with Kodak XAR5 X-ray film. Positive colonies were located, grown, and rescreened to isolate single positive colonies.

**Western transfer.** The identity of putative clones was confirmed after electrophoresis and transfer to nitrocellulose (2) by probing with affinity-purified antibodies and [125I]-labeled protein A.

**DNA sequencing.** DNA sequencing was performed (22) with the single-stranded M13 cloning vectors mp8 and mp9 (16). An oligonucleotide corresponding to amino acid residues 18 to 22 at the N terminus of the protein and having 16-fold redundancy was synthesized with an Applied Biosystems DNA synthesizer, model 381A, and used as a primer for sequencing.

### RESULTS

**Purification of pyruvate decarboxylase.** With cell extracts at pH 6.0, pyruvate decarboxylase does not bind to any of 60 dye-ligand columns when protein is loaded at 20 to 25 mg ml⁻¹ of column adsorbent⁻¹. However, some columns will bind up to 80% of the applied protein under these conditions; thus, passing through an appropriate dye adsorbent can achieve a fivefold purification. At pH 5.5 the enzyme will adsorb partly to several dyes and totally to Yellow H-E4R. It can be eluted from this dye by raising the pH, preferably with a simultaneous increase in ionic strength, which causes all bound proteins to be removed. A DEAE-Trisacryl or -Sephasel column subsequently separates the pyruvate decarboxylase from remaining contaminants. A summary of this procedure is given in Table 1.

The procedure is basically similar to that of Hoppner and Doelle (9), except that they did not achieve adsorption on a dye column, but only used a dye adsorbent to remove unwanted proteins. We have used several different dyes as the first, "negative" adsorbent, including Green H-4G and

<table>
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<tr>
<th>Enzyme sample</th>
<th>Protein (mg)</th>
<th>Pyruvate decarboxylase Activity (U)</th>
<th>Sp act</th>
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<tbody>
<tr>
<td>Extract</td>
<td>5,200</td>
<td>42,000</td>
<td>8</td>
</tr>
<tr>
<td>Not adsorbed on Green H-E4BD</td>
<td>1,250</td>
<td>40,000</td>
<td>32</td>
</tr>
<tr>
<td>Not adsorbed on Yellow H-E4R</td>
<td>170</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Eluted from Yellow H-E4R</td>
<td>550</td>
<td>38,000</td>
<td>72</td>
</tr>
<tr>
<td>Eluted from DEAE-Trisacryl</td>
<td>310</td>
<td>37,000</td>
<td>120</td>
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based (23). This gel was obtained by using total insert from the recombinant plasmid, recloned into the M13 sequencing vector, a sequence corresponding to nearly 200 bases of the 5' region was obtained by priming with an oligonucleotide designed to hybridize to the gene complementary to the sequence coding for amino acids at positions 18 to 22. The sequence included the region corresponding to amino acid 16 back to the start codon and 180 bases further upstream. To confirm the analysis, another oligonucleotide probe was made corresponding to the opposite strand to hybridize with bases -90 to -76 (numbering the first base of the start codon as zero). The full sequence is given in Fig. 3, in which possible RNA polymerase and ribosome-binding sites are indicated; a number of restriction endonuclease sites are also indicated.

**DISCUSSION**

Pyruvate decarboxylase is the key enzyme directing the production of CO₂ and ethanol as the end products of *Z. mobilis* fermentation.

The *Zymomonas* enzyme exhibits many properties similar to that from yeasts. Activity is lost if thiamine pyrophos-

N-terminal amino acid sequencing identified the first 35 amino acid residues. The sequence obtained is the following: N-Ser-Tyr-Thr-Val-Gly-Thr-Tyr-Leu-Ala-Glu-Arg-Leu-Val-Gln-Ile-Gly-Leu-Lys-His-His-Phe-Ala-Val-Ala-Gly-Asp-Tyr-Asn-Leu-Val-Leu-Asp-Asp-Leu . . . . . COOH.

Isolation of clones expressing *Z. mobilis* pyruvate decarboxylase in *E. coli*. Approximately 5,000 recombinant clones selected from the library were screened for expression of pyruvate decarboxylase, as described in Materials and Methods, in both the presence and the absence of IPTG. Several immunoreactive clones were detected, each with a different restriction pattern. One of these contained a strong band with the same electrophoretic mobility as *Z. mobilis* pyruvate decarboxylase (Fig. 2). This clone expressed pyruvate decarboxylase activity, in the absence of IPTG, at a level of 35 U mg⁻¹, more than four times the specific activity found in *Z. mobilis*. The strong protein band was also found to be antigenic to the affinity-purified antibodies as determined by Western transfer (data not shown) of extracts of host cells carrying the plasmid. No antigenic product was produced by host cells carrying the nonrecombinant plasmid pUC9.

**Sequence of the 5' region of the pyruvate decarboxylase gene.** By using the total insert from the recombinant plasmid, recloned into the M13 sequencing vector, a sequence corresponding to nearly 200 bases of the 5' region was obtained by priming with an oligonucleotide designed to hybridize to the gene complementary to the sequence coding for amino acids at positions 18 to 22. The sequence included the region corresponding to amino acid 16 back to the start codon and 180 bases further upstream. To confirm the analysis, another oligonucleotide probe was made corresponding to the opposite strand to hybridize with bases -90 to -76 (numbering the first base of the start codon as zero). The full sequence is given in Fig. 3, in which possible RNA polymerase and ribosome-binding sites are indicated; a number of restriction endonuclease sites are also indicated.

**Kinetic and structural properties of *Z. mobilis* pyruvate decarboxylase.** This preparation of *Z. mobilis* pyruvate decarboxylase was homogenous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with a subunit molecular size of 59,000 ± 1,000 daltons (Da) (Fig. 1). Gel filtration on a column of Sephacryl S-200 indicated a molecular size of 240,000 ± 5,000 Da in comparison with marker proteins. Its specific activity, measured at pH 6.5, was 110 to 130 U mg⁻¹.

The *Kₘ* for pyruvate was 0.3 mM, which is much lower than the reported value for the yeast enzyme (3), which shows allosteric behavior (11). A preparation of yeast enzyme (32) had a *Kₘ* of 1.2 mM and a Hill coefficient of 1.4 when assayed in the same conditions; the *Z. mobilis* enzyme had normal Michaelis-Menten kinetics. The pH optimum was found between 6.0 and 6.5 (Fig. 2), with no indication of a second peak of activity at lower pH, as reported elsewhere (8).

The *A₂₈₀* of a 1-mg/ml solution of the pure enzyme is 1.28, based on protein determination by the 205/280-nm method (23).

**FIG. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell extract (4 mg ml⁻¹) and purified *Z. mobilis* pyruvate decarboxylase (PDC). Lanes: 1, standard proteins (xanthine oxidase [150 kDa], phosphorylase b [96 kDa], bovine serum albumin [68 kDa], enolase [46 kDa], carbonic anhydrase [30 kDa], keto-deoxyporphoglucurate aldolase [22 kDa], and lysozyme [14 kDa]); 2, *Z. mobilis* cell extract; 3, purified *Z. mobilis* pyruvate decarboxylase (10 μg); 4, extract of *E. coli* control cells containing plasmid pUC9; 5, extract of *E. coli* cells expressing the cloned *Z. mobilis* pyruvate decarboxylase gene.

**FIG. 2.** pH profile of *Z. mobilis* pyruvate decarboxylase. The enzyme activity was determined by standard assay with 10 mM MES buffer adjusted by addition of acetate.
phate is omitted from the buffers, but can partially be recovered by preincubation with thiamine pyrophosphate. Activity is also irreversibly lost at pH values much above 7. The protein's tetrameric structure and size is like the yeast enzyme; on the other hand, there is no evidence either of two types of subunit (8) or of allosteric kinetic behavior. Two isoenzymes of yeast pyruvate decarboxylase have been described, one a homotetramer and both having Hill coefficients of >2 (11). The Zymomonas enzyme has a Hill coefficient of 1 and is a homotetramer. It has quite a high affinity for its substrate and an optimum pH close to physiological pH. The enzyme is relatively stable in an ammonium sulfate suspension and can be purified in large quantities when required.

Pyruvate decarboxylase in Z. mobilis makes up about 4% of the total protein, 2% of the biomass, and up to 10% of the protein readily extractable by gentle lysis. (These values vary somewhat with growth conditions.) With a specific activity of about 160 μmol min⁻¹ mg⁻¹ at 30°C, the activity in the cell is close to 3,000 μmol min⁻¹ g of biomass⁻¹. We have previously calculated that the flux through pyruvate decarboxylase in growing cells is 1,800 μmol min⁻¹ g of biomass⁻¹ (1). Thus, there is about 65% excess activity over the required amount, which may be needed to counteract inhibition by high concentration of the product CO₂ and perhaps by ethanol (17).

Notwithstanding the other features of a gene which influence transcription and translation, the level of expression of pyruvate decarboxylase in Z. mobilis implies that it has a highly efficient promoter. The results show unequivocally that we have selected a clone expressing the enzyme in E. coli from a Z. mobilis genomic library. Affinity-purified antibodies raised against the purified protein recognized a band of the same electrophoretic mobility as pyruvate decarboxylase as determined by Western transfer. The nucleotide sequence obtained from the clone was in agreement with the amino acid sequence of the amino terminus of the purified protein. Most important, E. coli extracts containing the recombinant clone express high levels of pyruvate decarboxylase in the presence and absence of IPTG, whereas E. coli lacks pyruvate decarboxylase activity.

Further experiments are required to confirm that the expression is totally under the control of the Zymomonas promoter as there remains the possibility that expression in the absence of IPTG may be due to incomplete repression of the lac promoter.

Gene expression systems, at least within gram-negative organisms, are to some extent compatible, so it would not be surprising that the Z. mobilis pyruvate decarboxylase gene can also be expressed very efficiently in E. coli. Other examples of expression in E. coli include the lysine pathway enzyme of Brevibacterium lactofermentum (14), Bacillus steatorrhophilus neutral protease (29), and Bacillus steatorrhophilus glyceraldehydedehyde phosphate dehydrogenase (4), the latter to a protein level approximately the same as reported here for Z. mobilis pyruvate decarboxylase.

The sequence of the 5' region of the Z. mobilis pyruvate decarboxylase gene (Fig. 3) contains a number of features. (i) The purine-rich region containing the putative ribosome-binding site GGAG is the characteristic 6 bases upstream of the start codon (28, 29). (ii) There is a possible polymerase-binding site which has the consensus last thymine of the Pribnow box at position -38. The sequence around -38 does not agree well with consensus sequences for the Pribnow box (20) (RNA polymerase binding site 2); only 3 of the bases agree with the sequence TATAAT (Fig. 4) (31). On the other hand, polymerase-binding site 1 of sequence ... GACACTTT ... occurs at the correct position, though again it does not contain all of the thymines that often occur immediately before the guanine of that sequence. A comparison of the Pribnow box with the consensus sequence recognized by E. coli RNA polymerase is made in Fig. 4.

Although E. coli strain JM101 does not grow well on glucose as sole carbon source, we are presently investigating the fermentation pattern of the recombinant clone containing pyruvate decarboxylase. It is to be expected that introduction of this enzyme (perhaps together with extra alcohol dehydrogenase) into another organism will direct its fermentation of sugars into production of ethanol. This has in fact been demonstrated with a Z. mobilis pyruvate decarboxylase clone, created with the cosmid vector pHC79 (5).

LITERATURE CITED


