Purification and characterization of pyruvate decarboxylase from *Sarcina ventriculi*

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Pyruvate decarboxylase from the obligate anaerobe *Sarcina ventriculi* was purified eightfold. The subunit M_r was $57\,000 \pm 3000$ as estimated from SDS-PAGE, and the native M_r estimated by gel filtration on a Superose 6 column was 240 000, indicating that the enzyme is a tetramer. The M_r values are comparable to those for pyruvate decarboxylase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*, which are also tetrameric enzymes. The enzyme was oxygen stable, and had a pH optimum within the range 6.3–6.7. It displayed sigmoidal kinetics for pyruvate, with a $S_{0.5}$ of 13 mM, kinetic properties also found for pyruvate decarboxylase from yeast and differing from the Michaelis–Menten kinetics of the enzyme from *Z. mobilis*. No activators were found. *p*-Chloromercuribenzoate inhibited activity and the inhibition was reversed by the addition of dithiothreitol, indicating that cysteine is important in the active site. The N-terminal amino acid sequence of pyruvate decarboxylase was more similar to the sequence of *S. cerevisiae* than *Z. mobilis* pyruvate decarboxylase.

Introduction

Pyruvate decarboxylase (EC 4.1.1.1) is a glycolytic enzyme catalysing the non-oxidative decarboxylation of pyruvate with the formation of acetaldehyde and carbon dioxide. Pyruvate decarboxylase is widespread in eukaryotes, including plants and yeast, but is relatively rare in prokaryotes, having been found only in *Sarcina ventriculi* (Stephenson & Dawes, 1971), *Zymomonas mobilis* (Hoppner & Doelle, 1983), *Acetobacter peroxydans* (De Ley & Schell, 1959), *Acetobacter suboxydans* (King & Cheldelin, 1954) and *Erwinia amylovora* (Haq & Dawes, 1971). Purification of pyruvate decarboxylase has been achieved from two micro-organisms, *Saccharomyces cerevisiae* (Ullrich *et al.*, 1966) and *Z. mobilis* (Hoppner & Doelle, 1983; Neale *et al.*, 1987a).

In *Sarcina ventriculi* a branch point for glucose fermentation occurs at pyruvate, where oxidative decarboxylation of pyruvate by pyruvate dehydrogenase takes place, leading to the formation of acetate, and non-oxidative decarboxylation by pyruvate decarboxylase results in ethanol production (Stephenson & Dawes, 1971). When grown at neutral pH, *S. ventriculi* forms equal levels of acetate and ethanol and lower levels of formate, whereas growth at pH 3.0 increases ethanol production (Lowe & Zeikus, 1991). Regulation of carbon and electron flow in *S. ventriculi* is achieved at the branch point for pyruvate, with increased levels of pyruvate decarboxylase relative to pyruvate dehydrogenase at low pH (Lowe & Zeikus, 1991).

Pyruvate decarboxylase from *S. ventriculi* was purified and characterized in order to compare its physical and chemical properties with the enzyme from *Sacch. cerevisiae* and *Z. mobilis*, and to provide information on the function of this enzyme in *S. ventriculi*.

Methods

Organism and culture conditions. *Sarcina ventriculi* strain JK was cultivated as described previously (Goodwin & Zeikus, 1987). For large-scale fermentation, 16-litre glass carboys were used with 12 litres of medium, and the culture was stirred by placing the vessel on a

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Abbreviation: TPP, thiamin pyrophosphate.

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magnetic stirrer. The pH of the medium was adjusted to pH 3.0 with 6.5 M-HCl prior to inoculation and the medium was reduced by the addition of 25 ml titanium/nitriilotriacetic acid solution (Moench & Zeikus, 1983). The cells were harvested during mid-exponential phase by centrifugation using a Sharples centrifuge (Pennsalt Chemicals Corporation, Philadelphia, PA, USA), and washed with distilled water, before being stored at -70°C .

Enzyme assay. Pyruvate decarboxylase activity was determined aerobically at 37°C in a 1 ml reaction mixture containing 0.1 M-sodium hydrogen maleate buffer (pH 6.5), 30 mM-pyruvate, 0.1 mM-thiamin pyrophosphate (TPP), 0.1 mM-MgSO₄, 0.3 mM-NADH and 15 units alcohol dehydrogenase. The wavelength and absorption coefficient for NADH were 340 nm and $6220\text{ M}^{-1}\text{ cm}^{-1}$, respectively.

Protein determination. Protein was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Purification of pyruvate decarboxylase. All steps were carried out under aerobic conditions at 4°C .

Step 1: Extraction and centrifugation. Cells of *S. ventriculi* (30 g) were suspended in 60 ml distilled water, and disrupted by one passage through a French pressure cell (American Instrument Co.) at 124 MPa. The cell debris was removed by centrifugation at 12000 g for 15 min, and the supernatant was used as the crude enzyme preparation.

Step 2: Ammonium sulphate fractionation. Solid (NH₄)₂SO₄ was added to the crude enzyme preparation initially at 30% saturation, with increases of 5% saturation until pyruvate decarboxylase was precipitated at 50% saturation. The precipitated material was resuspended in 0.1 M-piperazine/HCl buffer (pH 6.5) containing 1 mM-TPP and 1 mM-MgSO₄, and was washed and concentrated by ultrafiltration (YM 30 membrane; Amicon).

Step 3: Column chromatography. The above enzyme preparation was applied to a Q-Sepharose column (3.0 cm $\times$ 50 cm), previously equilibrated with 0.1 M-piperazine/HCl buffer (pH 6.5) containing 1 mM-TPP and 1 mM-MgSO₄. The column was washed with the same buffer and the enzyme eluted with a 0 to 1.0 M linear gradient of NaCl in the same buffer. Fractions containing pyruvate decarboxylase activity were pooled and concentrated by ultrafiltration. The sample was applied to a Sephacryl S-200 gel filtration column (3.0 cm $\times$ 50 cm) equilibrated with 0.1 M-piperazine/HCl buffer (pH 6.5) containing 1 mM-TPP, 1 mM-MgSO₄ and 0.1 M-NaCl. The fractions containing pyruvate decarboxylase were pooled and concentrated by ultrafiltration.

Electrophoresis and M_r determination. SDS-PAGE was performed as described by Laemmli (1970), with 12% (w/v) acrylamide. Protein bands were visualized by Coomassie brilliant blue G-250 staining.

M_r values of purified pyruvate decarboxylase were determined by gel filtration on a Superose 6 HR column (Pharmacia LKB Biotechnology) using a Waters FPLC system. The M_r markers used (Sigma) were Blue Dextran (M_r 2000000), as an estimate of the void volume of the

column, thyroglobulin (669000), apoferritin (443000), β -amylase (200000), alcohol dehydrogenase (150000) and BSA (66000). The M_r of the denatured protein was estimated by SDS-PAGE with low-range M_r standards (Bio-Rad): phosphorylase b (97000), BSA (66000), ovalbumin (43000), carbonic anhydrase (31000), soybean trypsin inhibitor (22000), and lysozyme (14000).

Amino acid sequence determination. The purified pyruvate decarboxylase was washed five times with double-distilled water using a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. The N-terminal amino acid sequence was determined by a protein sequencer model 477A (Applied Biosystems) with an on-line phenylthiohydantoin analyser (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Results and Discussion

Purification of pyruvate decarboxylase

Pyruvate decarboxylase was purified from *S. ventriculi* as outlined in Table 1. Column chromatography was carried out at pH 6.5, as lower pH values resulted in less enzyme adsorption and recovery from the column, and cation exchange was unsuccessful. The presence of the cofactors TPP and MgSO₄ was essential for enzyme stability. The enzyme was unstable at pH values above 7.5; instability of pyruvate decarboxylase at alkaline pH has also been reported for the enzyme from yeast (Gounaris *et al.*, 1971) and *Z. mobilis* (Neale *et al.*, 1987a), with the tetrameric form denaturing to monomers. It appears as a significant enzyme in cells of *S. ventriculi* grown at pH 3 (Fig. 1). The properties of pyruvate decarboxylase from *S. ventriculi* are shown in Table 2 and compared with properties of the same enzyme from *Z. mobilis* and *Sacch. cerevisiae*.

Pyruvate decarboxylase from *S. ventriculi* exhibits cooperative binding (Fig. 2), which has also been reported for the enzyme from *Sacch. cerevisiae* (Boiteux & Hess, 1970; Ullrich & Donner, 1970), and differs from the Michaelis-Menten kinetics found with pyruvate decarboxylase from *Z. mobilis* (Hoppner & Doelle, 1983; Neale *et al.*, 1987a).

Table 1. Purification of pyruvate decarboxylase from *S. ventriculi*

The data shown here are the mean results of three purifications.

Step	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Recovery (%)	Purification (-fold)
Crude enzyme extract	159	12800	12.9	100	1.0
(NH ₄) ₂ SO ₄ precipitation	54	9050	26.4	71	2.0
Q-Sepharose	5.6	1810	85	14	6.6
Gel filtration	2.7	724	103	6	8.0

Fig. 1. SDS-PAGE of M_r markers (lane 1), cell extract of *S. ventriculi*, 10 μ g (lane 2), and purified pyruvate decarboxylase, 4 μ g (lane 3).

Fig. 2. Kinetics of purified pyruvate decarboxylase for pyruvate. The data represent mean results from duplicate determinations, and are representative of data obtained from pyruvate decarboxylase purified on three separate occasions.

Table 2. Comparison of pyruvate decarboxylase from *S. ventriculi*, *Z. mobilis* and *Sacch. cerevisiae*

Characteristic	<i>S. ventriculi</i>	<i>Z. mobilis</i>	<i>Sacch. cerevisiae</i>
M_r (SDS)	57000 $\pm$ 3000	59000 $\pm$ 1000 ^{a,b}	60000 $\pm$ 3000 ^c
M_r (native)	240000	240000 $\pm$ 5000 ^b	230000 ^c
Specific activity [units (mg protein) ⁻¹]	103	120 ^b and 134.2 ^a	51.9 ^d
K_{cat} (min ⁻¹)	24720	32208	12456
$S_{0.5}$ or K_m for pyruvate (mM)	13	0.3 ^b and 4.4 ^a	3 ^e
pH optimum	6.3–6.7	6.0 ^b	5.4–5.8 ^f

References: a, Hoppner & Doelle (1983); b, Neale *et al.* (1987a); c, Gounaris *et al.* (1975); d, Ullrich *et al.* (1966); e, Ullrich & Donner (1970); f, Jordan *et al.* (1978).

The very low affinity for pyruvate of pyruvate decarboxylase from *S. ventriculi* could possibly have been due to the absence of a positive effector, for example a metal ion. The enzyme was incubated in 25 mM-EGTA at 37 °C for 20 h without loss of activity, suggesting that either metal ions were not required for activity or, if present, the association between the enzyme and the metal was so strong that EGTA could not remove the metal.

A number of inhibitors of *S. ventriculi* pyruvate decarboxylase were identified, including glyoxylate (2 mM caused 82% reduction in activity), which is an inhibitor of TPP-containing enzymes, forming a non-cleavable bond with the catalytic centre of TPP (Flatau *et al.*, 1988). Glyoxylate inhibits pyruvate synthase from *Clostridium kluyveri* and *Clostridium pasteurianum* (Thauer *et al.*, 1970), and pyruvate decarboxylase from *Sacch. cerevisiae* (Uhlemann & Schellenberger, 1976). Ethanol is the end product of the pathway for pyruvate metabolism and concentrations of 150 mM are formed

during the fermentation of glucose, but 100 mM-ethanol caused only a slight reduction (22%) in pyruvate decarboxylase activity. Phosphate increased the cooperativity and decreased the apparent affinity for pyruvate of pyruvate decarboxylase from *Sacch. cerevisiae* (Boiteux & Hess, 1970), but 100 mM-phosphate had no effect on pyruvate decarboxylase from *S. ventriculi*.

Pyruvate decarboxylase activity was inhibited by the presence of $ZnCl_2$, with 0.5 mM causing 82% reduction in activity; this inhibition was reversed by the addition of 5 mM-cysteine, which restored 65% of the total activity. Inhibition of pyruvate decarboxylase by *p*-chloromercuribenzoate (40 μ M resulted in 45% reduction in activity) occurred in the absence of pyruvate, whereas in the presence of pyruvate *p*-chloromercuribenzoate had no effect, indicating the importance of cysteine in the active site of this enzyme. The action of *p*-chloromercuribenzoate could be reversed by the addition of 5 mM-dithiothreitol. The metal ions Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} (each 2 mM) did not increase enzyme activity or alter the kinetic properties of pyruvate decarboxylase at pyruvate concentrations from 3 mM to 20 mM.

As none of the metal ions tested were positive effectors, a number of metabolic intermediates were examined to determine their effect on pyruvate decarboxylase activity. These included acetyl-CoA, CoA, acetyl phosphate, ATP, fructose 1,6-diphosphate and phosphoenol pyruvate, at varying concentrations up to 2 mM, and cAMP (14–100 μ M), with 9 mM-pyruvate. All the concentrations of acetyl-CoA (0.1 mM to 2 mM) caused approximately 27% loss in activity. Higher levels of acetyl phosphate (2 mM) and ATP (0.5 mM) decreased enzyme activity by 43% and 29%, respectively. The other metabolites did not alter pyruvate decarboxylase activity. It was not possible to ascertain the effect of

Fig. 3. The N-terminal amino acid sequence of purified pyruvate decarboxylase from *S. ventriculi* compared with the sequences of pyruvate decarboxylase from *Z. mobilis* and *Sacch. cerevisiae* using the Bestfit program of the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984). The boxed areas contain amino acids which directly match, : represents a conservative amino acid change.

Table 3. Relative amino acid composition of pyruvate decarboxylase from *S. ventriculi*, *Z. mobilis* and *Sacch. cerevisiae*

Amino acid	Composition (mol%)*		
	<i>S. ventriculi</i> ^a (this study)	<i>Z. mobilis</i> ^b	<i>Sacch. cerevisiae</i> ^c
Asx	8.7	10.2	10.2
Glx	10.7	8.6	8.9
Ser	5.5	4.2	6.0
Gly	7.3	8.1	7.8
His	1.1	2.1	2.0
Arg	4.1	3.0	2.7
Thr	6.1	4.6	7.7
Ala	7.2	15.0	8.2
Pro	2.7	4.8	4.6
Tyr	3.2	3.9	3.1
Val	6.8	7.8	7.5
Met	2.1	1.9	2.4
Ile	6.1	4.9	6.6
Leu	7.8	8.8	9.7
Phe	16.2	3.2	4.2
Lys	4.4	6.3	6.2
Cys	ND	1.2	1.1
Trp	ND	1.2	1.3
Total	100	99.8	100.2
Hydrophobic	48.9	45.3	49.2
Hydrophilic	43.8	46.4	43.2

ND, Not determined.

* References: a, this study; b, Neale *et al.* (1987b); c, Kellerman *et al.* (1986).

acetaldehyde on pyruvate decarboxylase activity as this compound is the substrate for the coupling assay with alcohol dehydrogenase.

The N-terminal amino acid sequence of pyruvate decarboxylase from *S. ventriculi* was compared to the sequences of *Z. mobilis* and *Sacch. cerevisiae* pyruvate decarboxylase (Fig. 3). There was 86% similarity and 54% identity in the N-terminal region of pyruvate decarboxylase from *S. ventriculi* compared with the enzyme from *Sacch. cerevisiae*, and 69% similarity and 44% identity between the *S. ventriculi* enzyme and the *Z. mobilis* enzyme. Comparison of the total amino acid composition of pyruvate decarboxylase from the three organisms (Table 3) did not reveal any great similarities. *Z. mobilis* pyruvate decarboxylase had a very high

content of alanine (Neale *et al.*, 1987b), and the enzyme from *S. ventriculi* was high in phenylalanine.

The amount of pyruvate decarboxylase protein increases when *S. ventriculi* is grown at low pH, corresponding to the increase in carbon and electron flow through the non-oxidative branch of the pathway for pyruvate metabolism (Lowe & Zeikus, 1991), suggesting that *in vivo* enzyme activity is not increased by altering the kinetics of the enzyme due to the presence of positive effectors, but rather by increasing the amount of protein synthesized. From a previous study using crude extract from cells of *S. ventriculi*, the K_m for pyruvate of pyruvate dehydrogenase was 2.5 mM (Lowe & Zeikus, 1991). As this enzyme has a much higher affinity for pyruvate than does pyruvate decarboxylase, increased flux through the non-oxidative branch of the pathway could be achieved by the presence of activators or increased synthesis of pyruvate decarboxylase. Our studies failed to identify any positive effectors, and increased levels of a protein with an M_r corresponding to pyruvate decarboxylase occur in cells grown at low pH compared to neutral pH (Lowe & Zeikus, 1991), suggesting that pyruvate decarboxylase synthesis rather than activity is increased when *S. ventriculi* is grown at acid pH.

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