Biochemistry and Physiology of Thiamin Diphosphate Enzymes

Proceedings of the International Meeting on the Function of Thiamin Diphosphate Enzymes

held in
Blaubeuren, October 1990

Edited by
Hans Bisswanger and Johannes Ullrich

VCH

Weinheim · New York · Basel · Cambridge

PYRUVATE DECARBOXYLASE FROM ZYMOMONAS MOBILIS: PROPERTIES OF THE ENZYME AND CATALYSIS OF C-C-BOND FORMATION

Stephanie Bringer-Meyer, and Hermann Sahm

Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, Postfach 1913, D-5170 Jülich, F.R.G.

SUMMARY

In the obligately fermentative bacterium *Zymomonas mobilis* pyruvate decarboxylase (EC 4.1.1.1) contributes 4-6 % of the soluble cell protein. The purified enzyme showed a number of similarities to the pyruvate decarboxylase from yeast, with respect to its molecular weight (200,000 ± 10,000), number of subunits (4 x 56,000 ± 4,000), and isoelectric point (4.9). Incubation of the enzyme at pH 8.9 resulted in a total loss of enzyme activity. The activity could be restored to 99.5 % of the initial value at pH 6.0 when both Mg^{2+} and thiamin pyrophosphate were present.

Since *Z. mobilis* contains 5 times more pyruvate decarboxylase activity than yeast the efficiency of this enzyme to catalyze acyloin formation was studied and compared to that of the yeast pyruvate decarboxylase. The pyruvate decarboxylases of both organisms catalyzed acetoin and phenylacetylcarbinol synthesis in the presence of pyruvate and acetaldehyde or benzaldehyde, but the affinity of the *Z. mobilis* pyruvate decarboxylase towards the aldehyde reactants was lower than that of the yeast enzyme. Because of the limited solubility of benzaldehyde, neither enzyme could be saturated with this substrate for phenylacetylcarbinol synthesis. Studies with 2-toluidinonaphthalene-6-sulfonate and substrate analogues showed that the catalytic sites of pyruvate decarboxylase from *Z. mobilis* were less lipophilic than those of the enzyme from yeast. This difference could explain the lower affinity for benzaldehyde of the *Z. mobilis* enzyme.

1 INTRODUCTION

Thiamin pyrophosphate-dependent pyruvate decarboxylase was first detected in yeast and has been found widely distributed in plants, but it has only a very limited occurence in bacteria (1,2,3). In the obligately fermentative bacterium *Zymomonas mobilis*, however, pyruvate decarboxylase plays a central role in the catabolism of glucose to ethanol and CO_2. This bacterium efficiently converts glucose via the Entner-Doudoroff pathway to pyruvate which is then decarboxylated by pyruvate decarboxylase to acetaldehyde which is reduced by alcohol dehydrogenase to ethanol (4). Since pyruvate decarboxylase can be regarded as a key enzyme in the ethanol formation by *Z. mobilis*, this enzyme was isolated and some of its molecular and catalytic properties were studied. In addition, the efficiency of C-C bond formation (Figure 1) of the *Z. mobilis* pyruvate decarboxylase, in the presence of acetaldehyde and benz-

aldehyde, was studied and compared to that of the pyruvate decarboxylase from yeast.

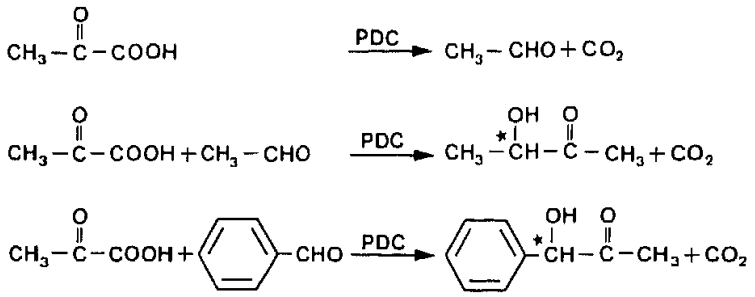

Figure 1. Reactions catalyzed by pyruvate decarboxylase (PDC) in the presence of pyruvate, acetaldehyde and benzaldehyde. Asterisks denote asymmetrical carbon atoms.

2 MATERIALS AND METHODS

2.1 *Microorganisms and Cultivation*

Z. mobilis, strain ATCC 29191 and *Saccharomyces carlsbergensis*, strain DSM 70424 were grown in non-aerated media which contained (g/100 ml): Bacto yeast extract (Difco, Detroit, MI, USA), 1.0; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0,1; $MgSO_4 \cdot 7H_2O$, 0.05; glucose, 5; pH 5.0. The growth temperature was 30°C.

2.2 *Preparation of Cell Extracts*

Cells from the late exponential growth phase were washed, suspended in potassium phosphate buffer (0.01 M, pH 6.0) and disrupted by sonication. The extract was centrifuged at 48,000xg and 5°C for 30 min. For the removal of low molecular weight components the supernatant was passed through a Sephadex G-25 column which was equilibrated and eluted with the above buffer. The resulting protein fraction contained 6-8 mg protein/ml and is referred to hereafter as cell-free extract.

2.3 *Chemicals and Enzymes*

Pyruvate decarboxylase from *Z. mobilis* was purified by differential $(NH_4)_2SO_4$ precipitation, and chromatographies on hydroxylapatite, DEAE cellulose, and Sephadex G-200 as reported previously (5). For studies on C-C bond formation additional purified pyruvate decarboxylase from *Z. mobilis* was kindly provided by Ms. G. Miczka, Institute of Enzyme Technology, University Düsseldorf. Pyruvate decarboxylase from *S. carlsbergensis* was a generous gift from Prof. Dr. J. Ullrich, Institute of Biochemistry, University Freiburg. Antibodies against the pyruvate decarboxylase from *Z. mobilis* were prepared as described by Bräu and Sahm (6). Phenylacetylcarbinol was obtained from Knoll AG, Ludwigshafen. All analytical enzymes were from Boehringer, Mannheim.

2.4 *Assay of Pyruvate Decarboxylase*

The enzyme activity was measured at 30°C. Prior to activity determinations the enzyme samples were diluted with sodium citrate buffer (0.1 M, pH 6.0, containing 20 mM Mg^{2+} and 1.5 mM thiamin pyrophosphate). The formation of acetaldehyde was determined by the method of Ullrich (7). For manometric assays of pyruvate decarboxylase, Warburg vessels contained sodium citrate buffer (480 µmol, pH 6.0), $MgSO_4$ (60 µmol), thiamin pyrophosphate (0.45 µmol), pyruvate (51 µmol) or other α-oxoacids (75 µmol) and limiting amounts of enzyme in a total volume of 3.0 ml. The reaction was started by addition of substrate from the side arm of the Warburg vessel. One unit of enzyme activity was defined as either the formation of 1 µmol acetaldehyde/min (also referred to as photometric units (PU) in the text) or the liberation of 1 µmol CO_2/min.

2.5 *Analytical Methods*

Protein was determined by the method of Bradford (8). For an estimate of the molecular weight (Stoke's radius) of the native enzyme a Sephadex G-200 column (d: 2.5 cm, length: 94 cm) was calibrated with calibration kits from Pharmacia, Freiburg, and Boehringer, Mannheim. Electrophoresis in the presence of sodium dodecylsulfate (SDS) was performed according to Laemmli (9). Calibration kits for SDS gel electrophoresis from Boehringer and Biorad, Munich, were used. Electrophoreses for the estimation of the purity of the enzyme were performed according to system 1 of Maurer (10). Polyacrylamide gel cylinders for isoelectric focusing were prepared by following the procedure of Righetti and Drysdale (11). Double immunodiffusion tests were conducted in 2 % agarose gels with a 0.05 M Tris-HCl buffer, pH 7.5, containing 0.025 % NaN_3 (12). Glucose and fructose concentrations were measured enzymatically, with hexokinase and glucose-6-phosphate dehydrogenase (13). Acetaldehyde and acetoin were analyzed by gas chromatography, with due precautions to prevent evaporative losses of acetaldehyde. The glass column (3.0 ft x 1/4 in) was packed with Porapak QS (80-100 mesh). Flow rate of the nitrogen carrier gas was 30 ml/min and temperatures of the injection port, column, and flame ionization detector were 250°, 110°, and 230°C, respectively. Benzaldehyde, benzyl alcohol, and phenylacetylcarbinol were determined by high pressure liquid chromatography. A reversed phase column (Spherisorb 5, ODS 1, 125 mm x 4 mm, CS-Chromatographie Service, Eschweiler) was eluted with a linear gradient of aqueous acetic acid (1 % v/v), and methanol containing 5 % (v/v) H_2O. Over a time interval of 15 min the ratio of methanol:acetic acid was increased from 10:90 to 55:45.

3 RESULTS

3.1 *Purification of Pyruvate Decarboxxylase*

After purification of the enzyme by differential $(NH_4)_2SO_4$ precipitation, and chromatographies on hydroxylapatite, DEAE cellulose, and Sephadex G-200 (Table 1), the enzyme preparation gave a single band upon polyacrylamide gel electrophoresis.

3.2 *Molecular and Kinetic Properties of the Enzyme*

3.2.1 *Molecular weight and subunits.*

The apparent molecular weight of the native pyruvate decarboxylase was estimated by gel chromatography (Sephadex G-200 column) to be 200,000 ± 10,000 which is slightly lower than the molecular weight of yeast pyruvate decarboxylase (230,000-250,000) (14) and of the enzyme from wheat germ (300,000) (15). When polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecylsulfate, only one protein band with a molecular

weight of 57,000 ± 4,000 was observed. These results suggest that the pyruvate decarboxylase from *Z. mobilis* is composed of four subunits of equal size, which are probably identical. The isoelectric point of the enzyme was determined by analytical isoelectric focussing to be 4.87.

Table 1. Purification of pyruvate decarboxylase from *Z. mobilis*. One unit (U) of enzyme is the amount that catalyzes the production of 1 µmol acetaldehyde/min.

Step	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (-fold)
Cell-free extract	1,045	11,408	100.0	10.92	1
$(NH_4)_2SO_4$ fraction 55-80 % saturation	813	10,199	89.4	12.55	1.2
Hydroxylapatite	106	7,067	61.9	66.62	6.1
DEAE cellulose	50	5,816	51.0	116.81	10.7
Sephadex G-200	14	2,580	22.6	181.00	16.6

3.2.2 *Cofactor dissociation and enzyme reconstitution.*

During the purification of the pyruvate decarboxylase from *Z. mobilis* the specific activity decreased after ammonium sulfate precipitations. Activity could be restored by the addition of Mg^{2+} and thiamin pyrophosphate to the dialyzed enzyme or by dialysis with buffer containing these cofactors. In order to determine the conditions for the reconstitution of the enzyme from *Z. mobilis* the dissociation and the association of the cofactors were studied (Table 2). Dialysis of an enzyme sample against a 0.08 M glycine - 0.2 M phosphate buffer containing 0.1 mM EDTA, and adjusted to pH 8.9 with KOH (7) resulted in a total loss of enzyme activity. After dilution of this sample into the assay buffer, pH 6.0, at 30°C the activity was restored time dependently, to 99.5 % when both Mg^{2+} and thiamin pyrophosphate were present. The low reactivation of 2.6 % of the initial enzyme activity which occured with Mg^{2+} or thiamin pyrophosphate alone could be due to traces of the cofactors in the dialyzed enzyme preparation.

Table 2. Conditions for the dissociation and association of the cofactors of pyruvate decarboxylase. TPP:thiamin pyrophosphate

Treatment	Incubation Mg^{2+} (20 mM)	Incubation TPP (1.5 mM)	Specific activity (U/mg protein)
Before dialysis	-	-	7.58
	+	+	8.18
Dialysis pH 8.9[a]	-	-	0.00
Dilution into pH 6.0[b]	+	+	8.14
	-	+	0.21
	+	-	0.21

[a] 0.08 M glycine - 0.2 M phosphate with 0.1 mM EDTA
[b] 0.1 M sodium citrate

3.2.3 *Affinity of the enzyme to the substrate and cofactors.*

An apparent K_m value for pyruvate of 0.4 mM was determined in the presence of 20 mM Mg^{2+} and 1.5 mM thiamin pyrophosphate in sodium citrate

buffer, pH 6.0.

The apparent K_m values of the apoenzyme for the cofactors Mg^{2+} and thiamin pyrophosphate were 24 µM and 1.28 µM. The apoenzyme was prepared by dialysis of an enzyme sample against the glycine-phosphate buffer described above. In order to reach association equilibria (16) the samples were incubated for 30 min at 30°C and pH 6.0 prior to activity measurements in the presence of limiting amounts of either Mg^{2+} or thiamin pyrophosphate and saturating concentrations of the second compound.

3.2.4 *Serological properties.*

The ability of antiserum prepared against the purified pyruvate decarboxylase from *Z. mobilis* to cross-react with pyruvate decarboxylases from other bacteria and from yeast was determined by Ouchterlony immunodiffusion tests. Surprisingly, no precipitation bands were formed with cell extracts of *Sarcina ventriculi*, *Erwinia amylovora*, or *Gluconobacter oxydans*, bacterial strains which are reportedly pyruvate decarboxylase positive (3), nor with cell extracts of *S. cerevisiae*. Therefore, it seems that the pyruvate decarboxylase from *Z. mobilis*, under the conditions used, is immunologically different from the other bacterial pyruvate decarboxylases and also from the yeast enzyme.

3.3 *Catalysis of Acyloin Formation by the Pyruvate Decarboxylases of Z. mobilis and S. carlsbergensis*

For quantitative comparison of acetoin synthesis by the pyruvate decarboxylases of *S. carlsbergensis* and *Z. mobilis*, acetoin formation as a function of the corresponding acetaldehyde concentrations was plotted (Figure 2). In the presence of low acetaldehyde concentrations (up to 8 mM) the *Z. mobilis* pyruvate decarboxylase produced only 1/10 of the acetoin formed by the yeast enzyme. It can be deduced from these results that the pyruvate decarboxylases of *Z. mobilis* and yeast differ in their K_m values for acetaldehyde whereas the maximal velocities of acetoin synthesis were found to be similar for both enzymes, i.e. when saturated with acetaldehyde.

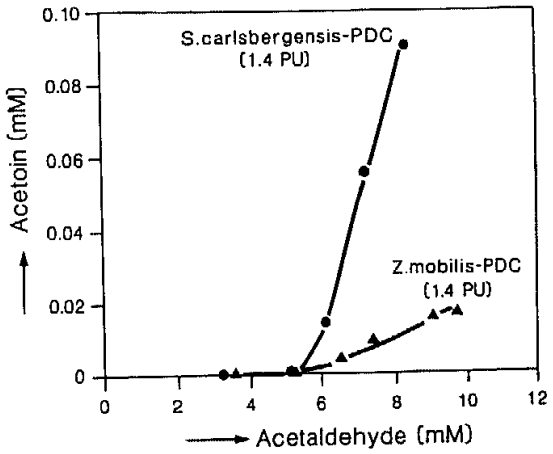

Figure 2. Comparison of acetoin synthesis by the pyruvate decarboxylases (PDC) of *S. carlsbergensis* and *Z. mobilis*.

With pyruvate and benzaldehyde as substrates phenylacetylcarbinol was synthesized by both pyruvate decarboxylases at increasing rates up to benzaldehyde concentrations of 46-48 mM (Figure 3). This behaviour indicates that neither enzyme was substrate-saturated with regard to benzaldehyde. However,

with 70 mM benzaldehyde the rate of phenylacetylcarbinol synthesis by the yeast pyruvate decarboxylase decreased drastically, presumably due to partial deactivation of the enzyme protein. The quantitative difference in phenylacetylcarbinol synthesis by the pyruvate decarboxylases of *Z. mobilis* and *S. carlsbergensis* was even more pronounced than that for acetoin synthesis (Figure 3).

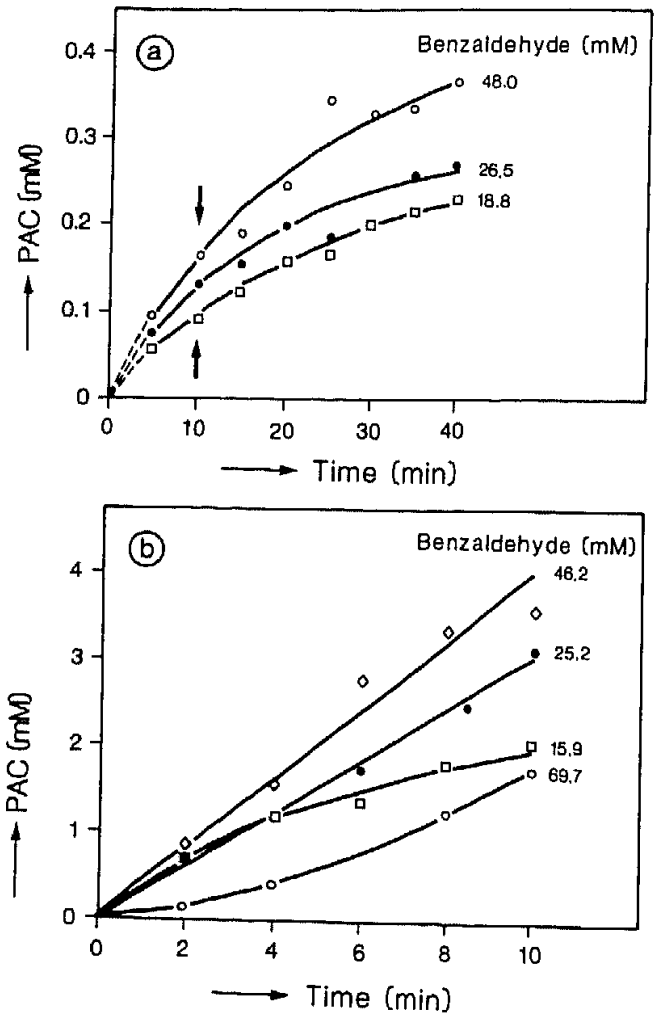

Figure 3. Dependence of phenylacetylcarbinol (PAC) synthesis on benzaldehyde concentration. Data for the pyruvate decarboxylases of *Z. mobilis* (8.1 PU, a) and *S. carlsbergensis* (8.0 PU, b). The arrows in (a) are for comparison of reaction times.

Thus, as already observed with acetaldehyde in acetoin synthesis, the two enzymes differ in their K_m values for benzaldehyde. Approximate K_m values for benzaldehyde of 50 and 125 mM for the yeast and *Z. mobilis* pyruvate decarboxylases, respectively, were estimated. Saturating conditions with respect to benzaldehyde could not be established since the solubility of this aromatic compound in aqueous solutions is not higher than 90-100 mM.

3.4 *Comparison of Active Site Lipophilicities of the Pyruvate Decarboxylases*

Yeast pyruvate decarboxylase possesses highly lipophilic substrate binding

sites, as was demonstrated by measurements with fluorescent dyes, e.g. 2-toluidinonaphthalene-6-sulfate (TNS) (17). The fact that the enzyme has relatively high affinities for α-oxoacids with longer alkyl chains than pyruvate (18) provides additional evidence. A strongly lipophilic zone is required in and around the active site of the enzyme to stabilize the α-carbanion of 2-α-hydroxyethylthiamin pyrophosphate over a sufficient lifetime for further conversion (19,14).

Fluorescent dyes like TNS exhibit much higher fluorescence yields in lipophilic environments than in aqueous solutions and tend to be bound exclusively to lipophilic areas (20). In the presence of yeast pyruvate decarboxylase the fluorescence of TNS was about 20 times higher than in protein-free buffer. By addition of pyruvate the fluorescence of TNS-pyruvate decarboxylase mixtures was reduced and inhibition experiments demonstrated a competitive inhibition of the enzyme by TNS (17). Thus, the K_i for TNS can be used as a measure for the lipophilicity of the catalytic site of the enzyme. The pyruvate decarboxylase from *Z. mobilis* was also found to be competitively inhibited by TNS; its inhibition constant (100 μM) however, was approximately 3 times higher than those reported for the enzymes from yeast (30 μM) or from wheat germ (39 μM) (21).

Determinations of the K_m values for α-oxobutyric acid with the *Z. mobilis* pyruvate decarboxylase gave a further indication that the binding sites of this enzyme are less lipophilic than those of the yeast enzyme (Table 3). With the manometric test employed for these measurements, the K_m value for pyruvate of 0.6 mM of the *Z. mobilis* pyruvate decarboxylase was not far different from the value of 0.4 mM which had been determined with the photometric test system, and was lower than that published for the pyruvate decarboxylase of yeast (Table 3). However, the bacterial enzyme showed a strongly decreasing affinity towards α-oxoacids with longer alkyl chains. This contrasts with the behaviour reported for the yeast enzyme, which exhibits higher or similar affinities for these α-oxoacids relative to pyruvate (18).

Table 3. Comparison of the K_m values for different α-oxoacids of the pyruvate decarboxylases from *Z. mobilis* and yeast. The numbers in parentheses give the values relative to pyruvate.

α-Oxoacid	K_m (mM) of pyruvate decarboxylases from *Z. mobilis*	Yeast (18)
Pyruvate	0.6 (1.0)	1.2 (1.0)
α-Oxobutyric acid	1.2 (1.9)	0.7 (0.6)
α-Oxocaproic acid	7.7 (11.9)	1.4 (1.2)

4 DISCUSSION

4.1 *Properties of Pyruvate Decarboxylase from Z. mobilis*

Pyruvate decarboxylase, a key enzyme in the ethanol formation of *Z. mobilis* channels 95 % of the substrate carbon via acetaldehyde to ethanol and CO_2, as can be calculated from fermentation balances (22). Correspondingly, this bacterium contains relatively high amounts of this enzyme which appears as a strong band when cell-free extracts are electrophoretically separated in polyacrylamide gels (result not shown). It can be calculated from the purification factor that under the conditions of cultivation used during this work, pyruvate decarboxylase contributes 4-6 % of the soluble cell protein. A comparison of specific pyruvate decarboxylase activities in different organisms is given in Table 4.

Table 4. Comparison of properties of pyruvate decarboxylases from different sources. TPP: thiamin pyrophosphate

Organism	Molecular weight	Molecular weight of subunits	K_m (pyruvate app.) (mM)	Spec.activity of cell-free extracts[a] (U/mg protein)	References
Z. mobilis	200,000	56,500	0.4	7.5-11.0	
S. cerevisiae	230-250,000	60,000	1.1	1.5-2.5	(23,24,7,14)
Wheat germ	275-300,000	75,000	3.6	0.02[b]	(15,25,14)

[a] Specific activities of cell-free extracts of *Erwinia amylovora*, *Sarcina ventriculi*, and *Gluconobacter suboxydans* were 1.1, 0.6, and 2.0 U/mg protein
[b] No photometrically determined values available; an estimated value from manometric data is given (25)

The activities of pyruvate decarboxylase reflect to some extent the efficiencies of the fermentative organisms to produce ethanol. In addition, the regulation of pyruvate decarboxylase and other pyruvate decarboxylating enzymes is decisive with respect to the pattern of fermentation products (26).

Although we could not detect an immunological cross-reaction between the pyruvate decarboxylases of yeast and *Z. mobilis*, these enzymes are similar in their molecular weights and subunit numbers (24). Furthermore, the conditions for the binding of the cofactors, thiamin pyrophosphate and Mg^{2+}, appear to be similar, if not identical, for both enzymes (16,14). A comparison of properties of pyruvate decarboxylases is shown in Table 4.

4.2 *Catalysis of C-C-bond Formation*

The comparison of acetoin and phenylacetylcarbinol syntheses by the pyruvate decarboxylases of *Z. mobilis* and *S. carlsbergensis* has shown that the bacterial enzyme has lower affinities than the yeast enzyme for the aldehyde reactants, acetaldehyde and benzaldehyde. In phenylacetylcarbinol synthesis from benzaldehyde and pyruvate this difference could not be overcome by establishment of saturating conditions with respect to benzaldehyde, since this aromatic compound has a limited solubility in aqueous solutions. Therefore, the rate of phenylacetylcarbinol synthesis by the *Z. mobilis* pyruvate decarboxylase was only 4-5 % of that of the yeast enzyme (Table 5). This difference in the rates was less pronounced when cell suspensions were used for the synthesis of phenylacetylcarbinol from sugar and benzaldehyde. The faster rate of sugar catabolism in *Z. mobilis* as compared to yeast, together with the 5-fold higher activity of pyruvate decarboxylase present in *Z. mobilis* cells, partially compensated the lower affinity of the *Z. mobilis* enzyme for benzaldehyde (Table 5).

Table 5. Comparison of *Z. mobilis* and *S. carlsbergensis* with respect to pyruvate decarboxylase activity and phenylacetylcarbinol formation.

Organism	Pyruvate decarboxylase $U \cdot (mg\ cell\text{-}free\ protein)^{-1}$	Synthesis of phenylacetylcarbinol by cell suspensions (mM)	by pyruvate decarboxylase (mM)
S. carlsbergensis	2	12.4	4.0
Z. mobilis	10	2.8	0.17

For both the main and side catalytic reactions of pyruvate decarboxylase

(decarboxylation and acyloin formation, respectively) the lifetime of the enzyme-bound reaction intermediate, 2-α-hydroxyethylthiamin pyrophosphate (HETPP), plays an important role. The rate-determining step in catalysis is the protonation of this carbanion followed by liberation of acetaldehyde (19). Thus, a relatively short lifetime of the HETPP-carbanion should favour decarboxylation of pyruvate to acetaldehyde and CO_2 and disfavour acyloin reactions with free aldehydes. In this connection it is noteworthy that the pyruvate decarboxylases purified from wheat germ and yeast have decarboxylation activities of 51 and 80 U/mg enzyme protein, respectively (27,28), whereas the activity of the purified *Z. mobilis* pyruvate decarboxylase was found to be 181 U/mg enzyme protein. This difference suggests that the HETPP-carbanion of the *Z. mobilis* enzyme has a shorter lifetime than that of the yeast enzyme. Such a view is further supported by results obtained with the competitive inhibitor, TNS, as well as with α-oxoacids, which are more lipophilic than pyruvate. Compared to the yeast pyruvate decarboxylase the K_i for TNS was about 3-fold higher; the K_m values for α-oxobutric and α-oxocaproic acid were significantly higher than that for pyruvate. Both results indicate that the catalytic sites of the *Z. mobilis* pyruvate decarboxylase are less lipophilic than those of the yeast enzyme, a situation which would explain the shorter lifetime of the HETPP-carbanion. It seems less probable that steric factors are responsible for the differences observed between the two decarboxylases since the enzyme from *Z. mobilis* had low affinities for both the bulky benzaldehyde and the relatively small acetaldehyde.

ACKNOWLEDGEMENTS

We wish to thank Dr. K.-L. Schimz for his help in the purification and characterization of pyruvate decarboxylase, Prof. R.K. Finn for valuable discussion, Dr. B. Sprey for his help in the preparation of antibodies, and Prof. H. Kneifel for his support in analytical problems. We thank Ms. M. Kerres and Ms. D. Rittmann for excellent technical assistance. Part of this research was supported by the Deutsche Forschungsgemeinschaft.

5 REFERENCES

1 C. Neuberg, L. Karczag. Biochem. Z. 36 (1911) 68-81
2 B. Vennesland in: P.D. Boyer, H. Lardy, K. Myrbäck (eds.), The Enzymes, vol. 2, Academic Press, New York, 1951, pp. 183-215
3 M.C. Scrutton. Methods Enzymol. 6A, (1971) 479-541
4 D.J. McGill, E.A. Dawes. Biochem. J. 125 (1971) 1050-1068
5 S. Bringer-Meyer, K.-L. Schimz, H. Sahm. Arch. Microbiol. 146 (1986) 105-110
6 B. Bräu, H. Sahm. Arch. Microbiol. 144 (1986) 296-301
7 J. Ullrich. Methods Enzymol. 18A (1970) 109-115
8 M.M. Bradford. Anal. Biochem. 72 (1976) 248-254
9 U.K. Laemmli. Nature 227 (1970) 680-685
10 H.R. Maurer in H.R. Maurer (ed.) Disc Electrophoresis, De Gruyter, Berlin/New York, 1971, pp. 41-52
11 P. Righetti, J.W. Drysdale. Biochim. Biophys. Acta 236 (1971) 17-28
12 D. Stollar, L. Levine. Methods Enzymol. 6 (1963) 848-854
13 H.U. Bergmeyer in: Methoden der enzymatischen Analyse, vol. 1, 3rd edn., Verlag Chemie Weinheim, 1974, pp. 502-503, 534-535
14 J. Ullrich. Ann. N.Y. Acad. Sci. 378 (1982) 287-305
15 H. Balla, J. Ullrich. Hoppe-Seyler's Z. Physiol. Chem. 361 (1980) 1265

16 A. Schellenberger, K. Winter, G. Hübner, R. Schwaiberger, D. Helbig, S. Schumacher, R. Thieme, G. Bouillon, K.-P. Rädler. Z. Physiol. Chem. 346 (1966) 123-147
17 J. Ullrich, I. Donner. Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 1030-1034
18 H. Lehmann, G. Fischer, G. Hübner, K.-D. Kohnert, A. Schellenberger. Eur. J. Biochem. 32 (1973) 83-87
19 A. Schellenberger. Angew. Chem. 79 (1967) 1050-1061; Internat. Ed. 6 (1967) 1024-1035
20 L. Brand, J.R. Gohlke. Ann. Rev. Biochem. 41 (1972) 843-868
21 H. Zehender. Dissertation, Universität Freiburg, FRG, 1984
22 P.L. Rogers, K.J. Lee, M.L. Skotnicki, D.E. Tribe. Adv. Biochem. Eng. 23 (1982) 37-84
23 A. Boiteux, B. Hess. FEBS Lett. 9 (1970) 293-296
24 A.D. Gounaris, J. Turkenkopf, L.L. Civerchia, J. Greenlie. Biochim. Biophys. Acta 405 (1975) 492-499
25 T.P. Singer, J. Pensky. J. Biol. Chem. 196 (1952) 375-388
26 R.K. Finn, S. Bringer, H. Sahm. Appl. Microbiol. Biotechnol. 19 (1984) 161-166
27 M. Sieber, S. König, G. Hübner, A. Schellenberger. Biomed. Biochim. Acta 42 (1983) 343-349
28 H. Zehender, D. Trescher, J. Ullrich. Eur. J. Biochem. 167 (1987) 149-154