Purification and characterization of 2-oxoglutarate decarboxylase of *Leuconostoc oenos*

RAINER KAPOL and FERDINAND RADLER*

Institut für Mikrobiologie und Weinforschung der Johannes Gutenberg-Universität Mainz, Postfach 3980, D-6500 Mainz, FRG

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2-Oxoglutarate decarboxylase from the lactic acid bacterium *Leuconostoc oenos* was purified by precipitation with PEG and ion-exchange chromatography. The strictly thiamin-pyrophosphate-dependent enzyme decarboxylated 2-oxoglutarate to succinic semialdehyde. Oxalacetate was metabolized to a lesser extent. The measured K_m values for 2-oxoglutarate and thiamin pyrophosphate were 1 mM and 0.03 mM respectively. The enzyme had a molecular mass in the range 65–70 kDa, did not consist of subunits and showed significant similarities to the corresponding mitochondrial enzyme of *Euglena gracilis*.

Introduction

Leuconostoc oenos is the main organism that causes the malolactic fermentation of wine. Besides decarboxylating malic acid, *L. oenos* metabolizes sulphur-dioxide-binding compounds such as acetaldehyde, pyruvate and 2-oxoglutarate (Mayer *et al.*, 1976). Whereas 2-oxoglutarate is usually reduced to 2-hydroxyglutarate by most lactic acid bacteria, a decarboxylation caused by *L. oenos* leads to the final products 4-hydroxybutyrate and succinate (Radler & Bröhl, 1984). A specific 2-oxoglutarate decarboxylase has been detected in cell extracts of *L. oenos* (Kapol *et al.*, 1990). This enzyme is presumed to decarboxylate 2-oxoglutarate to succinic semialdehyde. This rather unstable product is reduced by a dehydrogenase to 4-hydroxybutyrate in the presence of a hydrogen donor. In the absence of a hydrogen donor the final products are 2-hydroxyglutarate and succinate in a ratio of 1:1. The 2-oxoglutarate decarboxylase is dependent on TPP and is inducible by 2-oxoglutarate (Kapol *et al.*, 1990).

In this paper a method for the purification of 2-oxoglutarate decarboxylase from *L. oenos* B 211 is described and the main characteristics of this enzyme are reported.

Methods

Micro-organism and preparation of cell extracts. *L. oenos* strain B 211 was from the collection of this Institute. Methods for cultivation, preparation of cell extracts, and protein determination were as described previously (Kapol *et al.*, 1990).

Abbreviation: TPP, thiamin pyrophosphate.

Determination of the activity of 2-oxoglutarate decarboxylase. The carbon dioxide released from 2-oxoglutarate was measured manometrically at 30 °C. The main vessel contained: 0.1 ml cell extract; 0.1 ml thiamin pyrophosphate (TPP, 50 mM; pH 5.3); 2 ml buffer [composed of potassium phosphate buffer (100 mM, pH 5.3); magnesium sulphate (2 mM); dithiothreitol (1 mM); glycerol (30% v/v)]. To start the reaction 0.2 ml 2-oxoglutarate (0.5 M, pH 5.3) was added from the side arm. The reaction was stopped with 0.1 ml 7 M-sulphuric acid added from the second side arm. One unit (U) of 2-oxoglutarate decarboxylase is the amount required to catalyse the release of 1 $\mu\text{mol CO}_2 \text{ min}^{-1}$.

For the determination of the pH optimum and the stability of 2-oxoglutarate decarboxylase the following buffers (100 mM) were used: sodium citrate/disodium hydrogen phosphate (pH 3.5–6.0); potassium dihydrogen phosphate/disodium hydrogen phosphate (pH 5.2–8.2) and Tris/HCl (pH 7.1–8.7). To these buffers were added (final concentrations): MgSO_4 (5 mM); dithiothreitol (1 mM); TPP (4 mM), and glycerol (30%, v/v).

Purification of 2-oxoglutarate decarboxylase. To precipitate nucleic acids, protamine sulphate (salmine; 0.2 mg per mg protein) was added to the cell extract. After stirring for 10 min at 20 °C the sediment was removed by centrifugation (45 min, 40000 g, 4 °C). To 3 ml of the supernatant, 1 ml 50% (w/v) polyethylene glycol (PEG) 1500 was added and the mixture was vigorously shaken. After 18 h at 4 °C the sediment was collected by centrifuging (60 min, 40000 g) and dissolved in buffer solution [20 mM-potassium phosphate buffer, pH 6.7, containing 0.5 mM-dithiothreitol and 20% (v/v) glycerol]. This enzyme solution (about 6 ml) was applied to a column of DEAE-cellulose (23 SS Servacel) (2.6 × 26 cm) that had been equilibrated with buffer solution. Unbound proteins were washed out with 150 ml buffer solution. 2-Oxoglutarate decarboxylase was specifically eluted with 60–80 ml 20 mM potassium phosphate buffer, pH 6.3, containing 10 mM-2-oxoglutarate. 2-Oxoglutarate decarboxylase was the first protein to be eluted with this buffer. Only inactive proteins were eluted at pH values below 6.3.

Analytical methods. For isoelectric focusing the Rotoform system (Bio-Rad) was used. To the samples (220 mg protein in 20 ml), 20 mM-potassium phosphate buffer, pH 6.7, containing 30% (v/v) glycerol,

was added to give a total volume of 40 ml. Ampholine solutions (1.6 ml pH 3–5, and 0.4 ml pH 3–10) were added before focusing for 4.5 h at 4 °C with a current limited to a maximum of 12 W and 2000 V. Pharmacia equipment was used for SDS-PAGE (similar to the method of Weber & Osborn, 1969) and non-denaturing PAGE (as described by Caspritz & Radler, 1983). 2-Oxoglutarate and the reaction products were determined by HPLC (Pfeiffer & Radler, 1985).

Chemicals. Ampholines and protein molecular mass kits were from Pharmacia. 2-Oxoglutarate was obtained from Roth and Servacel DEAE-cellulose 23 SS was from Serva. All other chemicals, including PEG 1500, were purchased from Merck.

Results and Discussion

Purification of 2-oxoglutarate decarboxylase

From extracts of cells of *L. oenos* B 211 grown in the presence of 2-oxoglutarate, the inducible 2-oxoglutarate decarboxylase was purified 420-fold with a yield of 16%. The preparation was homogeneous on SDS-PAGE. A typical purification is presented in Table 1.

Characteristics of 2-oxoglutarate decarboxylase

Stability. In cell extracts to which glycerol (30%, v/v) and TPP (5 mM) had been added, the activity of 2-oxoglutarate decarboxylase remained unchanged for at least 6 d at 4 °C or several months at –20 °C. Purified preparations lost activity rapidly; after 24 h at 4 °C or –20 °C, activity loss was 95% and 25% respectively. When extracts adjusted to pH 5.3 were kept at 60 °C, only 10% of the activity was lost after 30 min, but 90% was lost after 5 min at 70 °C. When partially purified preparations of 2-oxoglutarate decarboxylase were kept at various pH values for 24 h at 4 °C, the enzyme was stable between pH 4.6 and 8.2.

Molecular mass. When the purified enzyme was subjected to PAGE a molecular mass of 70 kDa was calculated. With SDS-PAGE a molecular mass of 65 kDa was determined, indicating that the enzyme does not dissociate into subunits.

Substrate specificity. Whereas pyruvate, malate, lactate, propionate, tartrate and succinate were not substrates for the enzyme, oxalacetate was decarboxylated with a relative activity of 28% when compared with 2-oxoglutarate.

Kinetics. From a Lineweaver–Burk plot the K_m of 2-oxoglutarate decarboxylase for 2-oxoglutarate was determined as 1 mM. V was determined as $1.3 \mu\text{mol CO}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$.

Coenzymes. TPP was essential for the decarboxylation of 2-oxoglutarate. After dialysis for 6 h in 100 vols 100 mM-potassium phosphate buffer, pH 7.0, containing 30% (v/v) glycerol, and then in another 100 vols of the same buffer overnight at 4 °C, the extracts showed no activity. The activity of 2-oxoglutarate decarboxylase was restored by adding TPP (2 mM). This coenzyme could not be replaced by NADH, NAD, NADPH, NADP, ATP, ADP or CoA, nor did the addition of these compounds enhance the activity of 2-oxoglutarate decarboxylase in the presence of TPP. The K_m for TPP was 0.03 mM.

pH optimum and pI. 2-Oxoglutarate decarboxylase was active in the pH range 4.0–6.5 with an optimum activity at pH 5.3. By isoelectric focusing with the Rotofor the pI was found to be 4.2.

Influence of dibasic ions. None of the dibasic ions (1 mM) tested activated 2-oxoglutarate decarboxylase. The enzyme activity was not or only very little affected by Mg^{2+} , Mn^{2+} , Ca^{2+} , Ni^{2+} , Sn^{2+} or Ba^{2+} . The ions Co^{2+} , Zn^{2+} and Fe^{2+} caused an inhibition of up to 50%, whereas Cu^{2+} and Hg^{2+} inhibited the enzyme by 91% and 95% respectively.

Influence of SH reagents and chelators. 2-Oxoglutarate decarboxylase was activated by dithiothreitol (40%) and by mercaptoethanol (30%) at 5 mM. The SH antagonists *N*-ethylmaleimide and *p*-chloromercuribenzoate did not affect the enzyme activity. 2-Oxoglutarate decarboxylase was 84% inhibited by EDTA at a concentration of 5 mM, whereas *o*-phenanthroline caused an inhibition of 39%.

Table 1. Purification of 2-oxoglutarate decarboxylase from *L. oenos* B 211

Fraction	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Cell extract	484	76	0.16	1	100
Salmine sulphate precipitation	436	76	0.17	1.1	100
PEG 1500 precipitation	32	59	1.8	11	78
DEAE-cellulose 23 SS	0.18	12	66	420	16

General aspects

2-Oxoglutarate decarboxylase of *L. oenos* was rapidly inactivated in cell extracts but it could be stabilized by adding glycerol and TPP, enabling it to be stored at low temperature for several months. High losses of enzyme activity were encountered during the purification procedure and it was important to use as few steps as possible. The essential step was an unusual 'affinity elution' of 2-oxoglutarate decarboxylase from DEAE-cellulose 23 SS by slightly lowering the pH of the eluant and adding 2-oxoglutarate. This procedure led to a homogeneous enzyme preparation using only a few steps. It is not likely that 2-oxoglutarate caused a simple neutralization of the charged molecules, as has been observed with fructose-1,6-bisphosphate aldolase (Pogell, 1962) or fructose-1,6-bisphosphatase (Black *et al.*, 1972), for under the experimental conditions the enzyme and the substrate were negatively charged. It can be assumed that 2-oxoglutarate decarboxylase changes its conformational structure when 2-oxoglutarate is bound. This may alter its affinity for the column material. Such conformational changes affecting the interaction between protein and column material have been described with several enzymes (Bennet & Steitz, 1978; Pickover *et al.*, 1979).

The activity of 2-oxoglutarate decarboxylase was first observed in several strains of *L. oenos* (Radler & Bröhl, 1984). Subsequently this enzyme was detected in mitochondria of *Euglena gracilis* Z (Shigeoka *et al.*, 1986). The 2-oxoglutarate decarboxylases from *E. gracilis* and *L. oenos* have common characteristics: both enzymes are TPP specific; the end products of the decarboxylation of 2-oxoglutarate are succinic semialdehyde and CO₂; and oxalacetate is decarboxylated, but to a lesser extent than 2-oxoglutarate.

In *E. gracilis*, 2-oxoglutarate decarboxylase replaces the missing 2-oxoglutarate dehydrogenase complex, thus maintaining part of the tricarboxylic acid cycle. The biological significance of 2-oxoglutarate decarboxylase in *L. oenos* is not known. It can be assumed that the

reaction product succinic semialdehyde acts as a sink for hydrogen equivalents. This may be energetically favourable under anaerobic conditions. However, no significant increase in biomass is observed when 2-oxoglutarate is metabolized by *L. oenos* (F. Radler, unpublished). Most lactic acid bacteria reduce 2-oxoglutarate to 2-hydroxybutyrate; obviously 2-oxoglutarate dehydrogenase is missing in *L. oenos*.

References

- BENNET, W. S. & STEITZ, T. A. (1978). Glucose-induced conformational change in yeast hexokinase. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 4748–4752.
- BLACK, W. S., VAN TOL, A., FERNANDO, S. & HORECKER, B. L. (1972). Isolation of a highly active fructose diphosphatase from rabbit muscle: its subunit structure and activation by monovalent cations. *Archives of Biochemistry and Biophysics* **151**, 576–590.
- CASPRITZ, G. & RADLER, F. (1983). Malolactic enzyme of *Lactobacillus plantarum*. Purification, properties and distribution among bacteria. *Journal of Biological Chemistry* **258**, 4907–4910.
- KAPOL, R., STORCK, J. & RADLER, F. (1990). 2-Oxoglutarate decarboxylase of *Leuconostoc oenos*. *Folia Microbiologica* **35** (in the Press).
- MAYER, K., PAUSE, G. & VETSCH, U. (1976). Gehalte an SO₂-bindenden Stoffen in Wein: Einfluss von Gärung und biologischem Säureabbau. *Schweizerische Zeitschrift für Obst- und Weinbau* **112**, 309–313.
- PFEIFFER, P. & RADLER, F. (1985). Hochleistungsflüssig-chromatographische Bestimmung von organischen Säuren, Zuckern, Glycerin und Alkohol im Wein an einer Kationenaustauschersäule. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung* **181**, 24–27.
- PICKOVER, C. A., MCKAY, D. B., ENGELMAN, M. D. & STEITZ, T. A. (1979). Substrate binding closes the cleft between the domains of yeast phosphoglycerate kinase. *Journal of Biological Chemistry* **254**, 11323–11329.
- POGELL, B. N. (1962). Enzyme purification by selective elution with substrate from substituted cellulose columns. *Biochemical and Biophysical Research Communications* **7**, 225–230.
- RADLER, F. & BRÖHL, K. (1984). The metabolism of several carboxylic acids by lactic acid bacteria. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung* **179**, 228–231.
- SHIGEOKA, S., ONISHI, T., MAEDA, K., NAKANO, Y. & KITAOKA, S. (1986). Occurrence of thiamine pyrophosphate-dependent 2-oxoglutarate decarboxylase in mitochondria of *Euglena gracilis*. *FEBS Letters* **195**, 43–47.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.