JOURNAL OF BACTERIOLOGY, July 1992, p. 4838–4841
0021-9193/92/144838-04$02.00/0

Vol. 174, No. 14

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Isolation, Characterization, and Physiological Role of the Pyruvate Dehydrogenase Complex and α-Acetolactate Synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis

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Received 19 February 1992/Accepted 1 May 1992

The pyruvate dehydrogenase complex of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis has a specific activity of 6.6 U/mg and a K_m of 1 mM for pyruvate. The specific activities of E2 and E3 in the complex are 30 and 0.36 U/mg, respectively. The complex is very sensitive to NADH inhibition and consists of four subunits: E1α (44 kDa), E1β (35 kDa), E2 (73 kDa), and E3 (60 kDa). The *L. lactis* α-acetolactate synthase has a specific activity of 103 U/mg and a K_m of 50 mM for pyruvate. Thiamine pyrophosphate (K_m = 3.2 μM) and divalent cations are essential for activity. The native enzyme measures 172 kDa and consists of 62-kDa monomers. The role of both enzymes in product formation is discussed in view of NADH inhibition and competition for pyruvate.

Diacetyl is an essential aroma component in many dairy products. The main diacetyl-producing bacterium in these products is *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. The exact mechanism of diacetyl production by *L. lactis* is still a matter of discussion. Two bacterial mechanisms have been suggested: decarboxylation of α-acetolactate (3), which is formed by the α-acetolactate synthase (α-ALS) reaction, as found in many microorganisms (9, 14, 23); or direct synthesis from acetyl coenzyme A and acetyl-thiamine pyrophosphate (acetyl-TPP), as reported for *L. lactis* (12, 21). A third possibility is the condensation reaction of acetaldehyde and/or pyruvate, catalyzed by the pyruvate dehydrogenase complex (PDC) with hydroxyethyl-TPP as an intermediate product, as found in eukaryotes (11, 16). Recent data from experiments with whole cells (22, 24) and cell extracts of *L. lactis* (10) strongly suggest that the production of diacetyl and/or acetoin proceeds via α-acetolactate and that no diacetyl synthase is present. In view of the industrial importance of acetoin and diacetyl production by lactococci, it is important to determine the exact mechanism of production of these compounds. In this report we describe the isolation and characterization of the α-ALS of *L. lactis*. We describe the isolation of *L. lactis* PDC and our investigation of its role in diacetyl and acetoin production. Finally, we discuss the physiological role of both pyruvate-utilizing enzymes.

L. lactis subsp. *lactis* bv. diacetylactis C17, a culture from the Netherlands Institute for Dairy Research collection (10, 22), was grown in lactose-limited continuous culture at a dilution rate of 0.1 h^{-1} in diluted whey permeate medium (1.25% powder content) supplemented with 0.4% yeast extract and 0.2% Trypticase peptone (BBL, Cockeysville, Md.). Cell extracts were prepared by sonication after pretreatment with lysozyme (0.1%, 1 h, 37°C). The PDC was isolated essentially as described previously (20). A final high-performance liquid chromatography (HPLC)-gel filtration step was added (a Zorbax GF-250 column from Chrompack was used). α-ALS was isolated by protamine sulfate-ammonium sulfate precipitation followed by ion-exchange fast protein liquid chromatography with a Mono-Q column and then HPLC-gel filtration on a Zorback GF-250 column. α-ALS was purified in the presence of 20% glycerol to stabilize enzyme activity. The overall activities of the PDC (18), E2 (17), E3 (25), and α-ALS (9) were determined as described previously. Hydroxyethyl-TPP was synthesized from acetaldehyde and TPP as described by Gruys et al. (6), and protein was assayed as described by Bradford (1). The molecular weight of native protein was determined by HPLC-gel electrophoresis (Zorbax GF-250 column). The compositions and molecular weights of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The PDC was isolated to near homogeneity as judged from SDS-PAGE (Fig. 1). The subunit composition was the same as those of PDCs from other gram-positive bacteria (8, 20): E1α, E1β, E2, and E3, with apparent molecular masses on SDS-PAGE of 44, 35, 73, and 60 kDa, respectively. The isolation procedure (Table 1) resulted in the purification of 6 mg of PDC from 410 g (wet weight) of *L. lactis*. The total purification factor achieved for the PDC was 35-fold. Since the increases in specific activity were the same for E2 and E3, it was concluded that no factors were lost during purification. No ion-exchange steps were used during the purification, since this led to loss of E3 and inactivation of the PDC. Interestingly, this loss of activity could be com-

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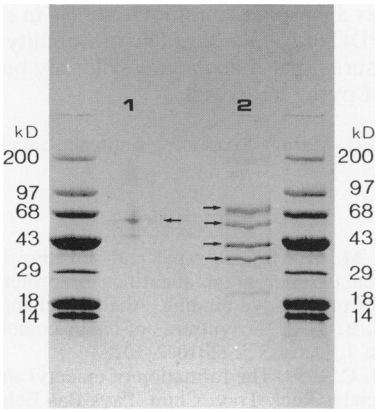

FIG. 1. SDS-PAGE of purified α-ALS (lane 1) and PDC (lane 2) of *L. lactis*. The arrows indicate the relevant polypeptides. The molecular mass markers are myosin (200 kDa), phosphorylase B (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa).

pletely reversed by the addition of excess pure E3 from *Enterococcus faecalis* (20). Saturation of the enzyme complex with pyruvate is shown in Fig. 2. From these data, a K_m for pyruvate of 1 mM and a Hill coefficient of 0.7 (Fig. 2, insert) were calculated. The PDC could utilize hydroxyethyl-TPP instead of pyruvate as an electron donor for NAD reduction, albeit at a reduced rate (40% activity with 40 mM hydroxyethyl-TPP compared with 100% activity with standard assay conditions). The K_m for TPP was 8.2 μM (data not shown). Inhibition of the complex by NADH showed competitive nonlinear characteristics that were the same as those for the PDC of *E. faecalis* (Fig. 3), but the PDC of *L. lactis* was much more sensitive than the PDC of *E. faecalis* to NADH. This, together with the low expression of E3 in *L. lactis*, explains why no in vivo PDC activity is observed anaerobically in *L. lactis*, in contrast to results with *E. faecalis* (19).

Isolation of α-ALS is described in Table 2. On SDS-PAGE gels (Fig. 1), the synthase appeared as one subunit of 62 kDa. The native α-ALS enzyme was found on gel filtration to have an apparent molecular mass of 172 kDa, suggesting that the synthase consists of several monomers. In aqueous solutions, α-ALS appeared to be sensitive to high salt concentrations (>0.2 M), especially at low protein concentrations. However, in 20% glycerol no loss of activity was observed after 2 months of storage at −20°C in purified form. Some kinetic parameters of the α-ALS were determined. α-ALS

TABLE 1. Purification of the PDC of *L. lactis*[a]

Sample	Vol (ml)	Amt of protein (mg)	PDC sp act (U/mg)	Total PDC activity (U)	Sp act (U/mg) of:	
					E2	E3
Cell extract	108	3,453	0.19	656	0.5	0.01
Protamine sulfate supernatant	119	2,279	0.38	866		
Polyethylene glycol pellet	3.5	876	0.62	543		
S-300	8.7	71	1.03	73	5.6	0.06
HPLC	10	6.1	6.55	40	30	0.36

[a] The starting material was 410 g (wet weight) of cells.

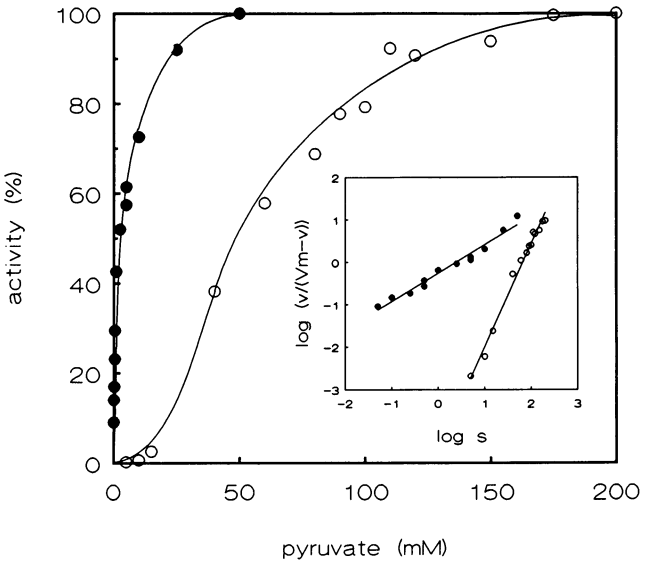

FIG. 2. Saturation kinetics for pyruvate of the PDC (●) and α-ALS (○) of *L. lactis*. The insert shows a Hill plot of the data.

had a pH optimum of 6.0 and showed Michaelis-Menten dependency for TPP (K_m = 3.2 μM). Activity was completely inhibited by EDTA (5 mM) but could be reversed by adding Mg^{2+} or Mn^{2+} ions. α-ALS could not use hydroxyethyl-TPP (together with pyruvate) as a substrate for the synthesis of acetolactate. Instead, hydroxyethyl-TPP acted as a competitive inhibitor (K_i = 30 μM) for TPP. The purified enzyme was neither stimulated by the addition of FAD (1.5 mM) nor subject to feedback control from the branched-chain amino acid valine (1.5 mM), leucine (1.5 mM), or isoleucine (1.5 mM), as found in anabolic α-ALSs (4, 5, 13). The α-ALS of *L. lactis* showed a strong positive cooperativ-

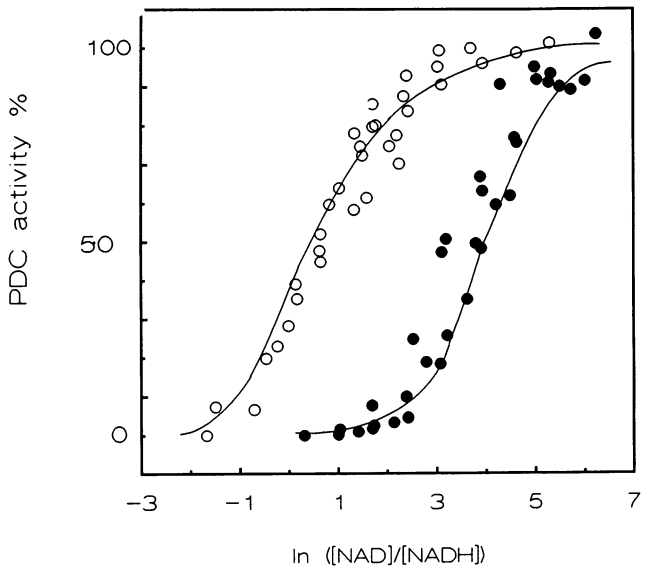

FIG. 3. Relationship between ln([NAD^+]/[NADH]) and activity of the PDCs of *E. faecalis* (○) and *L. lactis* (●). Activity is expressed as the percentage of the activity found at the same NAD^+ concentration with no NADH added.

TABLE 2. Isolation of the α-ALS of *L. lactis*[a]

Sample	Vol (ml)	Amt of protein (mg)	Sp act (U/mg)	Total activity (U)
Cell extract	19	931	2.1	1,950
Ammonium sulfate precipitate (40%)	7.5	594	2.8	1,649
FPLC[b]	13	17	28	480
HPLC	11	1.3	103	132

[a] The starting material was 70 g (wet weight) of cells.
[b] FPLC, fast protein liquid chromatography.

ity in the binding of pyruvate (n = 2.4 in a Hill plot [Fig. 2]), with an extremely low affinity for pyruvate (K_m = 50 mM). This aspect plays an important role in the regulation of acetoin and diacetyl production. The production of these C_4 compounds is only observed under conditions of high internal pyruvate accumulation, as is the case during citrate fermentation at low culture pH (10, 22). This observation has led to the suggestion that the production of C_4 compounds is a means of removing toxic pyruvate (2), especially at low pHs. The kinetic data, and the high specific activity and constitutive high expression of α-ALS support this view. This provides *L. lactis* with a powerful mechanism for keeping the internal pyruvate concentration within limits, while assuring that no competition between the α-ALS and enzymes such as lactate dehydrogenase, pyruvate formate lyase, and the pyruvate dehydrogenase complex will occur.

With the purified PDC, no acetolactate or acetoin synthesis could be measured with pyruvate and/or acetaldehyde as a substrate. During the isolation of the PDC and α-ALS, copurification of both enzymes was observed (in the protamine sulfate precipitation step). This suggested that both enzymes form a specific association. Indeed, it could be that, under conditions in which the PDC is limited by E3 activity (as is likely to be the case in the PDC of *L. lactis* at elevated levels of NADH), hydroxyethyl-TPP, which could be used by α-ALS, is accumulated. However, when both purified enzymes were mixed, no binding of the enzymes was observed on HPLC-gel filtration and no stimulation of either α-ALS or PDC activity was observed. Instead, total separation of PDC and α-ALS was achieved by final gel filtration without significant loss of overall activity of either enzymes. Furthermore, the characteristics of both enzymes were completely different with respect to affinity for pyruvate and subunit composition. Finally, the enzymes were not simultaneously expressed; the PDC reached maximal activity in aerobic, lactose-limited cultures and was virtually absent in anaerobic batch cultures, whereas α-ALS was constitutively expressed under all of these conditions (data not shown). It is clear now that the PDC and α-ALS are two completely different enzymes with different physiological functions and that only the latter is involved in α-acetolactate production. This enzyme, in pure form, catalyzes the TPP-dependent synthesis of one molecule of α-acetolactate from two molecules of pyruvate. The TPP dependency is also evident from the presence of the TPP-binding structural motif (7) in the primary structure, as deduced from the nucleotide sequence of *L. lactis* α-ALS (15).

From the determined characteristics of the purified PDC and α-ALS, it can be concluded that the NADH/NAD ratio and the internal pyruvate concentration play an important role in regulation of the catabolic fluxes over these enzymes. The high sensitivity of the PDC for NADH prevents in vivo activity under anaerobic conditions; this is in contrast to the case of the PDC of *E. faecalis*. The low affinity of α-ALS for pyruvate ensures that this enzyme will only be active under conditions of pyruvate excess.

We thank J. D. Marugg for sharing sequence data before publication.

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