JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 757–767
0021-9193/91/020757-11$02.00/0

Vol. 173, No. 2

Purification and Characterization of Acetoin:2,6-Dichlorophenolindophenol Oxidoreductase, Dihydrolipoamide Dehydrogenase, and Dihydrolipoamide Acetyltransferase of the *Pelobacter carbinolicus* Acetoin Dehydrogenase Enzyme System

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Dihydrolipoamide dehydrogenase (DHLDH), dihydrolipoamide acetyltransferase (DHLTA), and acetoin: 2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR) were purified from acetoin-grown cells of *Pelobacter carbinolicus*. DHLDH had a native M_r of 110,000, consisted of two identical subunits of M_r 54,000, and reacted only with NAD(H) as a coenzyme. The N-terminal amino acid sequence included the flavin adenine dinucleotide-binding site and exhibited a high degree of homology to other DHLDHs. DHLTA had a native M_r of >500,000 and consisted of subunits identical in size (M_r 60,000). The enzyme was highly sensitive to proteolytic attack. During limited tryptic digestion, two major fragments of M_r 32,500 and 25,500 were formed. Ao:DCPIP OR consisted of two different subunits of M_r 37,500 and 38,500 and had a native M_r in the range of 143,000 to 177,000. In vitro in the presence of DCPIP, it catalyzed a thiamine pyrophosphate-dependent oxidative-hydrolytic cleavage of acetoin, methylacetoin, and diacetyl. The combination of purified Ao:DCPIP OR, DHLTA, and DHLDH in the presence of thiamine pyrophosphate and the substrate acetoin or methylacetoin resulted in a coenzyme A-dependent reduction of NAD. In the strictly anaerobic acetoin-utilizing bacteria *P. carbinolicus*, *Pelobacter venetianus*, *Pelobacter acetylenicus*, *Pelobacter propionicus*, *Acetobacterium carbinolicum*, and *Clostridium magnum*, the enzymes Ao:DCPIP OR, DHLTA, and DHLDH were induced during growth on acetoin, whereas they were absent or scarcely present in cells grown on a nonacetoinogenic substrate.

The ability of bacteria to degrade acetoin is widespread among aerobic bacteria, and the catabolism of acetoin had been investigated 35 years ago (25). *Acinetobacter calcoaceticus* degrades acetoin via the 2,3-butanediol cycle (25). In contrast, other bacteria such as *Bacillus subtilis* (31) and *Alcaligenes eutrophus* (19, 56) catalyze oxidative cleavage of acetoin into two C_2 carbon compounds, as do bacteria that rely on a fermentative metabolism, such as *Streptococcus faecalis* (13) and *Pelobacter carbinolicus* (36). However, little biochemical work has been done yet, and the mechanism of acetoin cleavage is still unknown.

The strict anaerobe *P. carbinolicus* Gra Bd 1 utilizes only 2,3-butanediol, methylacetoin, acetoin, and ethylene glycol as substrates for growth (36). Since this bacterium ferments acetoin to equimolar amounts of acetate and ethanol, it lends itself to study the anaerobic acetoin-degradative pathway. Recent studies provided strong evidence that oxidative-thiolytic cleavage of acetoin into acetyl coenzyme A (acetyl-CoA) and acetaldehyde and of methylacetoin into acetyl-CoA and acetone occur in this bacterium during growth on acetoin and 2,3-butanediol or methylacetoin, respectively (36). *P. carbinolicus* synthesizes acetoin:2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR), dihydrolipoamide dehydrogenase (DHLDH), and dihydrolipoamide transacetylase (DHLTA) during growth on acetoin, 2,3-butanediol, and methylacetoin but not on ethylene glycol. In addition, acetyl-CoA was detected upon oxidative cleavage of methylacetoin or of acetoin. Therefore, cleavage of acetoin might occur by a reaction that is analogous to the oxidative-thiolytic cleavage of pyruvate to acetyl-CoA and CO_2 catalyzed by the pyruvate dehydrogenase multienzyme complex (37).

In this study, we analyzed the cells of five other strict anaerobic bacteria, which had been grown on acetoin and on a nonacetoinogenic substrate, for the presence of Ao:DCPIP OR, DHLDH, and DHLTA. Also in these bacteria, formation of the enzymes mentioned above was induced during growth on acetoin. Besides the catabolism of glycine, which is currently under investigation (11, 12, 17), the catabolism of acetoin provides a second example for involvement of DHLDH in anaerobic metabolism. DHLDH, DHLTA, and Ao:DCPIP OR were purified from acetoin-grown cells of *P. carbinolicus*, and the properties of these enzymes were compared with those of homologous enzymes from other organisms. By combining Ao:DCPIP OR, DHLTA, and DHLDH, the putative physiological acetoin-cleavage reaction was reconstituted.

MATERIALS AND METHODS

Bacterial strains. *P. carbinolicus* Gra Bd 1 (DSM 2380; 45), *Pelobacter propionicus* Ott Bd 1 (DSM 2379; 45), *Pelobacter venetianus* Gra PEG1 (DSM 2394; 49), *Pelobacter acetylenicus* WoAcy 1 (DSM 3246; 47), *Acetobacterium carbinolicum* Wo Prop 1 (DSM 2925; 15), and *Clostridium magnum* WoBdP 1 (DSM 2767; 46) were obtained from the Deutsche Sammlung für Mikroorganismen (DSM).

Growth conditions. The basal mineral medium for *P. propionicus*, *P. venetianus*, and *P. acetylenicus* had the

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following composition per liter: K_2HPO_4, 0.2 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.13 g; NaCl, 1.0 g; KCl, 0.5 g; and trace solution SL 7, 1 ml (63). For cultivation of *P. carbinolicus*, the composition of the medium was identical except that the NaCl concentration was 20 g/liter. During preparation, the media were kept under an atmosphere of 80% (vol/vol) nitrogen and 20% (vol/vol) carbon dioxide. The media were reduced with 0.5 g of cysteine hydrochloride per liter. The pH was adjusted to 7.2 by adding solid $NaHCO_3$. The mineral medium for *A. carbinolicum* was prepared as described before (15). *C. magnum* was cultivated in chopped meat medium (described in the 1989 DSM Catalogue of Strains).

Filter-sterilized carbon sources were added to the autoclaved media as indicated in the text. Whereas *P. carbinolicus* was cultivated at 37°C, all other bacteria were grown at 30°C. The cells were harvested by 20 min of centrifugation at 10,000 × *g* and resuspended in buffer. Mass cultivation was done in 5- or 20-liter carboys. Growth was monitored photometrically at 650 nm.

Preparation of crude extract. The cells were disrupted by a twofold passage through a French press at 96 MPa. Crude extracts were obtained by 50 min of centrifugation at 100,000 × *g* and 4°C.

Enzyme assays. DHLDH (EC 1.8.1.4.) was assayed photometrically at 365 nm in 90 mM potassium phosphate buffer, pH 7.2, in the presence of 2 mM EDTA, 0.2 mM NADH, and 2 mM lipoamide (37, 60). The reaction was started by addition of lipoamide. Stock solutions of lipoamide were prepared in dimethyl sulfoxide (11). DHLTA (EC 2.3.1.12) was measured with a UV spectroscopic assay at 240 nm (50). For *S*-acetyldihydrolipoamide, an ε_{240} of 1.46 mM^{-1} cm^{-1} was determined according to the hydroxamate assay as described by Reed and Willms (43). DL-Dihydrolipoamide was prepared by reduction of DL-lipoamide as described before (42). Ao:DCPIP OR was assayed photometrically at 578 nm as described previously (36). Alcohol dehydrogenase (EC 1.1.1.1), 2,3-butanediol dehydrogenase (EC 1.1.1.4), malate dehydrogenase (EC 1.1.1.37), and acetate kinase (EC 2.7.2.1) were determined by measuring changes in A_{365} nm with NAD and NADH, respectively (4, 57). Acylating acetaldehyde dehydrogenase (EC 1.2.1.10) was measured by monitoring the increase in A_{340} nm in 100 mM imidazole hydrochloride, pH 7.5, in the presence of 1 mM acetaldehyde, 1 mM NAD, 1 mM coenzyme A, and 1 mM dithiothreitol; the reaction was started by addition of coenzyme A (10). Phosphotransacetylase (EC 2.3.1.8) was determined spectroscopically at 233 nm (45). All assays were performed at 30°C. One unit of enzyme activity is defined as the transformation of 1 μmol of substrate per min.

Purification of DHLDH. A 50 mM imidazole hydrochloride buffer, pH 7.2, was used throughout the purification procedure. A crude extract (ca. 1 g of protein) derived from about 16 g (wet weight) of cells was dialyzed for 3 h and then applied onto a column (2.6 by 11 cm; 58-ml bed volume [BV]) of DEAE-Sephacel equilibrated with the buffer. After the column was washed with 2 BV of buffer, the protein was eluted with a linear KCl gradient (0 to 500 mM; two times, 150 ml) at a flow rate of 0.5 BV/h. Fractions containing high enzyme activity were combined, concentrated, and washed by ultrafiltration in a Diaflo chamber, using a PM 30 membrane. The enzyme solution was applied onto a column (2.6 by 4 cm; 21-ml BV) of procion brown MX-5BR–Sepharose (2), which had been equilibrated with buffer. After the column was washed with 3 BV of buffer, the protein was eluted with a linear KCl gradient (0 to 600 mM; two times, 500 ml) at a flow rate of 3 BV/h. Fractions containing high enzyme activity were pooled, concentrated, and washed by ultrafiltration with 100 mM Tris hydrochloride, pH 7.2. Finally, the enzyme solution was heated to 75°C for 10 min. Precipitated protein was removed by 5 min of centrifugation at 11,000 × *g*. DHLDH was recovered from the supernatant.

Purification of DHLTA. A 50 mM Tris–10 mM EDTA buffer, pH 7.5, was used throughout the purification procedure. The buffer contained phenylmethanesulfonyl fluoride (PMSF), leupeptin, and pepstatin at concentrations of 1 mM, 1 μM, and 1 μM, respectively. The protease inhibitors were added from stock solutions immediately before the buffer was used. Stock solutions were prepared as follows: PMSF, 100 mM in 2-propanol; leupeptin, 2 mM in H_2O; and pepstatin, 2 mM in methanol.

Crude extract (ca. 0.5 g of protein) derived from about 8 g (wet weight) of cells was dialyzed for 3 h against buffer and then applied onto a column (2.6 by 4.5 cm; 24-ml BV) of DEAE-Sephacel equilibrated with the buffer. After the column was washed with 2 BV of buffer, the protein was eluted with a linear KCl gradient (150 to 600 mM; two times, 120 ml) at a flow rate of 0.5 BV/h. Fractions containing high enzyme activity were combined and concentrated by ultrafiltration in a Diaflo chamber, using a PM 30 membrane. The enzyme solution was desalted by a passage through a PD 10 column and was subsequently applied onto a Mono Q HR5/5 column (1-ml BV), which was connected to an LCC-500-controlled FPLC apparatus (Pharmacia-LKB Biotechnologie GmbH, Freiburg, Federal Republic of Germany). After the column was washed with 2 BV of the buffer, the protein was eluted with a linear KCl gradient (200 to 1,500 mM, with a concentration change of 15 mM KCl/ml) at a flow rate of 1 ml/min. Fractions with high enzyme activity were combined and concentrated by ultrafiltration. Salts were removed by a passage through a PD 10 column.

Purification of Ao:DCPIP OR. A buffer consisting of 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MOPS), pH 7.2, and 10 mM $MgCl_2$ was used throughout. Crude extract (ca. 1.0 g of protein) derived from about 16 g (wet weight) of cells was dialyzed for 4 h against buffer and then applied onto a column (2.6 by 11 cm; 58-ml BV) of DEAE-Sephacel equilibrated with buffer. After the column was washed with 2 BV of buffer, proteins were eluted with a linear KCl gradient (0 to 400 mM; two times, 250 ml) at a flow rate of 0.5 BV/h. Fractions with high enzyme activity were combined, concentrated by ultrafiltration (PM 30), and desalted (PD 10). The enzyme solution was applied onto a column (2.6 by 10 cm; 53-ml BV) containing a mixture of Matrex gel blue A, procion red HE-3B–Sepharose, and procion brown MX-5BR–Sepharose (2:1:1, wt/wt). Ao:DCPIP OR was eluted with 2.5 BV of buffer. After concentration and desalting, the protein solution was finally applied onto a Mono Q HR5/5 column. After the column was washed with 2 BV of buffer, Ao:DCPIP OR was eluted with a linear KCl gradient (0 to 300 mM, with a concentration change of 15 mM KCl/ml) at a flow rate of 1 ml/min. Fractions with high enzyme activity were combined, concentrated by ultrafiltration, and desalted by a passage through a PD 10 column.

Molecular weight determinations by gel filtration. A Superose 12 column (1.0 by 30 cm; 24-ml BV; Pharmacia LKB Biotechnologie) was equilibrated with 50 ml of buffer. Purified enzymes and calibration proteins (1.0 mg each) were applied onto the column with a flow rate of 0.5 ml/min. Relative molecular masses were calculated from semilogarithmic plots of the molecular masses of calibration proteins against elution volume.

TABLE 1. Purification of DHLDH from *P. carbinolicus*

Step	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	33.0	35.6	1,175	647	0.55	1	100
Dialysis	35.0	31.6	1,106	613	0.55	1	94.7
DEAE-Sephacel	13.5	14.3	193	493	2.55	4.6	76.2
Procion brown	15.5	1.2	18.6	405	21.8	39.6	62.6
Heat precipitation	6.8	2.1	14.4	370	25.7	46.7	57.2

Electrophoretic methods. Native polyacrylamide gel electrophoresis (PAGE) was performed under nondenaturing conditions with 4% (wt/vol) and 7% (wt/vol) gels in a flat gel apparatus for vertical slab electrophoresis with 125 mM Tris–19 mM borate buffer, pH 8.9 (55). Linear polyacrylamide gradient gels (5 to 27.5%, wt/vol) were run at a constant power of 7 W for 48 h (1). Sodium dodecyl sulfate (SDS)-PAGE was performed either in 7.5% (wt/vol) gels as described by Weber and Osborn (62) or in 11.5% (wt/vol) gels as described by Laemmli (30).

Activity staining of gels for DHLDH was done in the presence of NADH, lipoamide, and 5,5′-dithiobis(2-nitrobenzoic acid) or 5-nitroblue tetrazolium chloride as described previously (37).

Analysis of metabolites. Ethanol, acetaldehyde, acetate, acetone, acetoin, 2,3-butanediol, methylacetoin, and diacetyl were determined by gas chromatography as described before (57).

Flavin identification. The enzyme-bound flavin was liberated by thermal denaturation of the protein at 100°C for 15 min in the dark and centrifugation for 15 min at 15,000 × *g* (11). Flavin was separated by high-performance liquid chromatography (HPLC) on a reverse-phase column (Nucleosil 100 C18) in an apparatus provided by Knauer KG (Bad Homburg, Federal Republic of Germany) at a flow rate of 2.0 ml/min. Flavins were eluted with a linear gradient of 10 to 40% (vol/vol) methanol in aqueous 5 mM ammonium acetate, pH 6.0 (34), and detected by measuring A_{254}. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and riboflavin were used as references. For quantitative determination of FAD, an extinction coefficient $\varepsilon_{450} = 11.3$ mM^{-1} cm^{-1} was used (52).

Spectroscopic methods. Absorption spectra of DHLDH were obtained anaerobically under an atmosphere of nitrogen at room temperature with a Lambda 3 UV/VIS double-beam spectrophotometer (Perkin Elmer Bodenseewerk, Überlingen, Federal Republic of Germany). Samples were measured against a reference containing the sample buffer.

N-terminal sequence analysis. Sequence analysis was performed with a 477A pulsed liquid-phase protein/peptide sequencer (21) and a 120A on-line PTH amino acid analyzer (44) (both from Applied Biosystems, Weiterstadt, Federal Republic of Germany) according to the instructions of the manufacturer. At positions where no signal was obtained, cysteine may have been the amino acid present, since the protein was not carboxymethylated before being sequenced.

Determination of protein. Soluble protein was determined as described by Lowry et al. (33).

Immunological methods. Antisera against purified DHLDH and DHLTA were raised in rabbits (New Zealand, female, 3 months old, 2 kg) after intramuscular injections of approximately 1 mg of protein suspended in complete Freund adjuvant. Three weeks later, subcutaneous booster injections of 0.5 mg of protein suspended in incomplete Freund adjuvant were made. After 2 more weeks, the rabbits were bled. Immunoglobulins G (IgGs) were purified from the serum by chromatography on protein A–Sepharose CL-6B (23). Double-immunodiffusion tests were performed as described before (39). For identification of antigens in the protein pattern of crude extract of *P. carbinolicus*, electroblots were performed with a semidry Fast Blot B33 (Biometra GmBH, Göttingen, Federal Republic of Germany) at constant 5 mA/cm^2 according to the instructions of the manufacturer. Antigenic proteins were stained with anti-rabbit IgG alkaline phosphatase conjugate (59).

Chemicals. CoA, cytochrome *c*, FMN, and anti-rabbit IgG alkaline phosphatase conjugate were obtained from Sigma Chemie (Deisenhofen, Federal Republic of Germany); all other coenzymes and enzymes were obtained from C. F. Boehringer & Söhne (Mannheim, Federal Republic of Germany); relative molecular mass standard proteins and Desulfo-CoA Agarose were from Sigma; HMW calibration kit for molecular weight determinations, DEAE-Sephacel, lysine–Sepharose 4B, and protein A–Sepharose CL-6B were from Pharmacia (Freiburg, Federal Republic of Germany); procion brown MX-5BR and procion red HE-3B were from Deutsche ICI (Frankfurt, Federal Republic of Germany); Matrex gel blue A was from Amicon Corp. (Lexington, Ky.); polyvinylidene fluoride membranes were obtained from Millipore (Bedford, Mass.); DL-lipoamide, DL-lipoic acid, acrylamide, *N*,*N*′-methylenebisacrylamide, imidazole, Tris, SDS, and *N*,*N*,*N*′,*N*′-tetramethylethylethylenediamine were purchased from Serva Feinbiochemica (Heidelberg, Federal Republic of Germany); methylacetoin was from Aldrich-Chemie (Steinheim, Federal Republic of Germany); and acetoin and 2,3-butanediol were from Fluka Chemie (Buchs, Switzerland).

RESULTS

Purification of DHLDH. The DHLDH from acetoin-grown cells of *P. carbinolicus* was purified 47-fold to electrophoretic homogeneity in a three-step procedure (Table 1). After cell breakage, 75% of DHLDH activity was detected in the supernatant of a 100,000 × *g* centrifugation. Chromatography on DEAE-Sephacel resulted in a 4.6-fold increase of the specific activity, with 76% recovery of the enzyme activity. The enzyme preparation obtained was tested with eight different triazine dye affinity media. DHLDH bound to Matrex gel blue A, procion red HE-3B–Sepharose, and procion brown MX-5BR–Sepharose; the enzyme did not bind to Matrex gel orange A, Matrex gel green A, procion yellow MX-8G, procion blue MX-2G, and procion green HE-4BD. Because of the strength of binding, procion brown MX-5BR–Sepharose was chosen for further purification. DHLDH was eluted from the column at 125 mM KCl and yielded a yellow enzyme solution. The enzyme was enriched 8.5-fold in this step, and 82% of the activity was recovered.

Because of the considerable insensitivity to high temperatures exhibited by DHLDH of *P. carbinolicus*, contaminating proteins were finally removed by heat precipitation. SDS-PAGE and native PAGE of the purified enzyme revealed only one band in each case. The purified DHLDH remained stable for months if stored in liquid nitrogen.

Properties of DHLDH. SDS-PAGE of the final DHLDH preparation resulted in one protein band, indicating the presence of only one type of subunit with an M_r of 54,000 ± 1,000. In contrast, the nondenatured protein exhibited an M_r of 110,000 ± 5,000 in gradient gel electrophoresis. These results indicate that the native DHLDH is a dimer of two identical subunits.

The DHLDH from *P. carbinolicus* proved to be highly specific for NAD(H) as a coenzyme; no reaction was observed with NADP(H). One typical property of DHLDHs is the variety of physiological and artificial electron acceptors which are used in vitro (60, 64). This was also found for the enzyme isolated from *P. carbinolicus*. Besides lipoamide (activity = 100%), high reductase activities were measured if lipoamide was replaced by ferricyanide (31.1%), menadione (13.6%), DCPIP (12.3%), methylene blue (9.4%), 5-nitroblue tetrazolium chloride (5.1%), or benzyl viologen (2.9%) as the electron acceptor in the standard assay. Activities with methyl viologen, FAD, FMN, or cytochrome *c* were less than 0.5%.

For the NADH:lipoamide oxidoreductase activity, which is the putative physiological reaction, the following K_m values were calculated from Lineweaver-Burk plots: K_m (NADH), 0.15 mM; K_m(lipoamide), 3.7 mM; K_m(NAD), 0.62 mM; and K_m(dihydrolipoamide), 0.40 mM.

The pH optimum for the NADH-dependent reduction of lipoamide was at 7.2 in potassium phosphate buffer as well as in Tris hydrochloride or imidazole hydrochloride buffer (each 100 mM). The highest rate was obtained with potassium phosphate buffer; with Tris hydrochloride and imidazole hydrochloride buffers, only 87 and 79%, respectively, of the maximum rate were measured.

The absorption spectrum of purified DHLDH from *P. carbinolicus* showed the characteristics of flavoproteins, with one maximum at 455 nm and one shoulder at 481 nm (34, 35, 51, 64). Anaerobic titration of the oxidized enzyme with NADH resulted in a stepwise decrease in absorbancy around 455 nm (Fig. 1). The flavin component was liberated by heat treatment and was identified as FAD by HPLC on a reverse-phase column, which separates FAD from FMN and riboflavin. From a calibration curve obtained with this method and from the measurement of A_{450}, FAD contents of 0.71 and 0.82 mol/mol of subunit, respectively, were calculated. From these data, we conclude that the DHLDH of *P. carbinolicus* contains one noncovalently bound FAD per subunit.

The N-terminal amino acid sequence of DHLDH from *P. carbinolicus* exhibited a high degree of homology to DHLDHs of different origin (Table 2), including the FAD-binding site (position 13 to 18), the sequence of which is known from other pyridine nucleotide-disulfide oxidoreductases (7, 12).

Purification of DHLTA. The DHLTA of acetoin-grown cells of *P. carbinolicus* was purified 24-fold to electrophoretic homogeneity in a two-step procedure (Table 3). Because of the high sensitivity of DHLTA from *P. carbinolicus* to proteolytic digestion (see below), the purification of DHLTA was performed in the presence of the protease inhibitors EDTA, PMSF, leupeptin, and pepstatin (29).

After cell breakage, 88% of DHLTA activity was detected in the supernatant of a 100,000 × *g* centrifugation. Chromatography of a dialyzed supernatant on DEAE-Sephacel resulted in an 8.0-fold increase of the specific activity, with 39% recovery of the enzyme activity. Purification of DHLTA was finally achieved by chromatography on Mono Q HR. In this step, the enzyme was enriched 3.0-fold and 72% of the activity was recovered. Both SDS-PAGE and native PAGE of the purified enzyme revealed only one band (Fig. 2A, lane 1; Fig. 2B, lane 4).

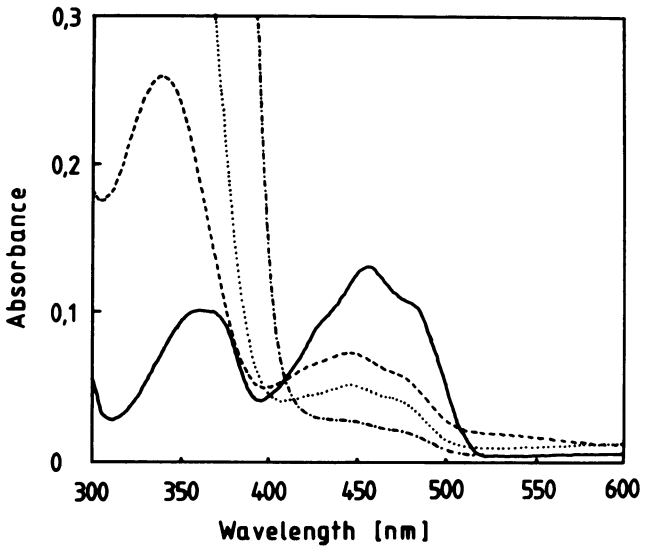

FIG. 1. Absorption spectra of the DHLDH from *P. carbinolicus*. Spectra were taken anaerobically under an N_2 atmosphere in 100 mM Tris hydrochloride, pH 7.2, from the purified enzyme (0.94 mg/ml). The oxidized enzyme (———) was stepwise reduced by addition of NADH: – – –, after 1.5 mol of NADH/mol of enzyme; · · · ·, after 3 mol of NADH/mol of enzyme; · - · - · -, after 15 mol of NADH/mol of enzyme.

Properties of DHLTA. SDS-PAGE resulted in a single protein band, indicating the presence of only one type of subunit with an M_r of 60,000 ± 1,000. The molecular weight of the native enzyme could not be determined exactly either by linear 5 to 27.5% gradient PAGE or by gel filtration on Superose 12 (Pharmacia LKB Biotechnologie). However, from the data obtained from these experiments, it can be concluded that the M_r of native DHLTA from *P. carbinolicus* is higher than 500,000.

Dihydrolipoamide acyltransferases (E_2 components) of 2-oxo acid dehydrogenase complexes from different sources are very sensitive to proteolytic cleavage under nondenaturing conditions (5, 9, 20, 28, 40). After limited proteolysis, a domain comprising the lipoyl moieties (designated the lipoyl-binding domain) and a domain containing the acetyltransferase activity (designated the catalytic or subunit-binding domain) are usually obtained. Time-limited incubation of purified DHLTA from *P. carbinolicus* in the presence of 0.45 μg of trypsin per ml resulted in a decrease of the portion of the intact protein and the appearance of two major fragments of M_r 28,500 and 32,500 and of one minor fragment of M_r 52,500, as revealed by SDS-PAGE (Fig. 2A). During digestion, aliquots were taken and tested for DHLTA activity. The enzyme activity was retained during the incubation period without significant loss. Preparations of DHLTA obtained in the absence of protease inhibitors (Fig. 2A, lane 7) exhibited a protein pattern which was very similar to the

TABLE 3. Purification of DHLTA from *P. carbinolicus*

Step	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	17.8	32.5	578.5	12,131	21.0	1	100
Dialysis	20.5	25.5	522.8	10,970	21.0	1	90.4
DEAE-Sephacel	4.2	7.1	29.8	4,784	168.5	8.0	39.4
Mono Q HR	4.0	1.7	6.8	3,461	509.0	24.2	28.5

pattern of the tryptic digest of structurally intact DHLTA (Fig. 2A, lane 2 to 5).

The pH optimum for the acetyl-CoA:dihydrolipoamide transfer reaction was at 6.7 in Tris hydrochloride as well as in imidazole hydrochloride or potassium phosphate buffer (each 100 mM). Highest rates were obtained in Tris hydrochloride buffer; in imidazole hydrochloride and potassium phosphate buffers, only 82 and 66%, respectively, of the maximum rate were obtained. For dihydrolipoamide, a K_m value of 2.2 mM was calculated from a Lineweaver-Burk plot. Neither covalently protein-bound dihydrolipoic acid, which is probably the physiological state of the acetyl carrier, nor CoASH or their acetylated derivatives are accessible to kinetic determinations by the test assays used in this study (43, 50), which measure only a part of the reverse reaction of the putative physiological acetyl transfer (6).

Amino acid sequence analysis of the intact enzyme revealed the following N-terminal sequence: 1-(Ser)-Asp-Asn-Arg-Ile-(Ile)-Ala-Leu-Thr-Met-Pro-Lys-(Lys)-Gly-Leu-Thr-Met-Glu-Glu-Gly-Leu-Ile-22.

Purification of Ao:DCPIP OR. Purification of active Ao:DCPIP OR from acetoin-grown *P. carbinolicus* to electrophoretic homogeneity was achieved in a three-step procedure (Table 4). To stabilize the enzyme, 10 mM $MgCl_2$ was added to the buffer. Removal of DHLDH, which coeluted with Ao:DCPIP OR during chromatography on DEAE-Sephacel, and of other proteins was achieved by passage through a column containing a mixture of three different DHLDH-binding triazine dye affinity media. Purification was finally achieved by chromatography on Mono Q HR; 3.5% of the activity could be recovered during the purification procedure, resulting in a 1.8-fold increased specific activity. Electrophoresis of the purified enzyme in native polyacrylamide gels and in native gradient gels revealed only one band. No further loss of activity occurred if the enzyme was stored in liquid nitrogen in the presence of 50% (vol/vol) glycerol.

Properties of Ao:DCPIP OR. In SDS-polyacrylamide gels, two distinct bands were detected, indicating two different subunits with similar relative M_rs of 38,500 ± 1,000 and 37,500 ± 1,000 (Fig. 3), which were designated α subunit and β subunit, respectively. Gradient gel electrophoresis and gel filtration on Superose 12 revealed relative M_rs of 143,000 ± 5,000 and 177,000 ± 10,000, respectively, for the native protein.

Ao:DCPIP OR was detected in various bacteria if the cells were cultivated on acetoin (31, 36, 56). In the presence of the artificial electron acceptor DCPIP, Ao:DCPIP OR from *P. carbinolicus* catalyzes a thiamine pyrophosphate (TPP)-dependent oxidative-hydrolytic cleavage of acetoin, methylacetoin, and diacetyl according to the equations shown below, as revealed by gas chromatographic analysis:

TABLE 2. Comparison of N-terminal sequence data of DHLDH from *P. carbinolicus* with those of other DHLDHs

Organism	Sequence[a]	Reference
Homo sapiens	1 A D Q P I D A D V T V I G S G P G G Y V A A I K A A Q L G F K T V C 34	38
Escherichia coli	1 S T E I K T Q V V V L G A G P A G Y S A A F R C A D L G L E T V I V E R Y N T 39	58
Peptostreptococcus glycinophilus	1 S K H F D(S)I V L G A G P G G Y V A A I K G A Q L G L N V A I V(E)? R 35	12
Clostridium sporogenes	1 K D I I V I G G G P G G Y V A A I R A A H L G A D V A V V(E)M D S 33	12
Clostridium cylindrosporum	1 S Q I Y D V I V I G G G P A G Y V G A I K A A Q L G G K V A I V(E)K D N 36	12
Pelobacter carbinolicus	1 A D E I F D L I V L G A G P G G Y V G A I R A A Q L G M K V A V V ? S ? P 37	This study
Consensus sequence	d v v v 1 G g G P g G Y v a A i k a a q L G t v v e	12
	FAD-binding site	

[a] ( ), Identification tentative; ?, no identification; Boldface, identical with DHLDH from *P. carbinolicus*, as determined by Genetics Computer Group sequence analysis.

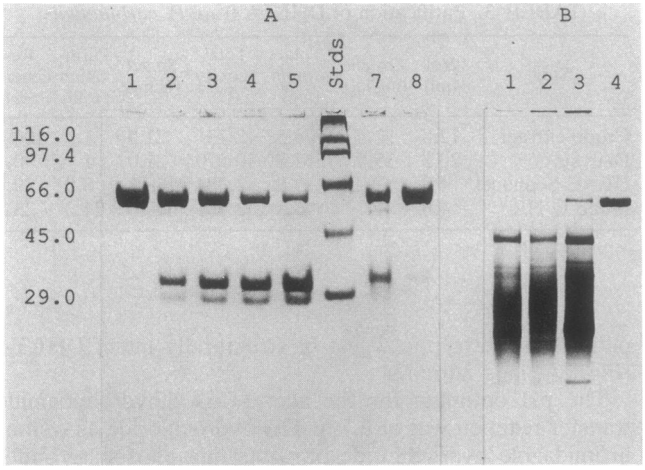

FIG. 2. PAGE of DHLTA from *P. carbinolicus*. (A) Time course of limited tryptic digestion of purified enzyme. Samples were applied to 7.5% (wt/vol) SDS-polyacrylamide gels as described in Materials and Methods. DHLTA (1.7 mg/ml) was incubated with trypsin (0.45 μg/ml) in 50 mM Tris hydrochloride buffer–10 mM EDTA, pH 7.5, in an ice bath. Digestion was started by addition of trypsin; aliquots (15 μl) were taken immediately before (lane 1) and 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), and 40 min (lane 5) after addition of trypsin; proteolysis was stopped by 3 min of incubation at 95°C in the presence of 2% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol. Lane 7, Protein pattern of DHLTA purified in the absence of protease inhibitors; lane 8, after 45 min of tryptic digestion of DHLTA in the presence of 1 mM PMSF. Molecular masses of standards (Stds) are given on the left in kilodaltons. (B) Comparison of the protein pattern of ethylene glycol-grown and of acetoin-grown cells of *P. carbinolicus* with purified DHLTA. Samples were applied to native 4% (wt/vol) polyacrylamide gels as described in Materials and Methods. Lanes: 1 and 2, 135 μg of protein from crude extracts of different ethylene glycol-grown cultures; 3, 165 μg of protein from crude extract of acetoin-grown cells; 4, 52 μg of purified DHLTA. Protein was stained with Serva Blue R.

$$CH_3COC(OH)CH_3 + DCPIP + H_2O \rightarrow CH_3CHO + CH_3COOH + DCPIPH_2$$

$$CH_3COC(CH_3)_2OH + DCPIP + H_2O \rightarrow CH_3COCH_3 + CH_3COOH + DCPIPH_2$$

$$CH_3COCOCH_3 + DCPIP + 2H_2O \rightarrow 2CH_3COOH + DCPIPH_2$$

The relative activities as measured by monitoring the reduction of DCPIP were 100, 124, and 41% with acetoin, methylacetoin, and diacetyl, respectively. No activity was measured with pyruvate or with 2-oxoglutarate as the substrate. Formation of diacetyl did not occur during DCPIP-dependent oxidation of acetoin. No reduction of the following electron acceptors was observed in the presence of acetoin: NAD, FAD, FMN, cytochrome *c*, ferricyanide, and viologen dyes. For Ao:DCPIP OR, K_m values of 7.7 and 10.5 μM were calculated from double-reciprocal Lineweaver-Burk plots for acetoin and TPP, respectively. The pH optimum was at 7.2 in 100 mM imidazole hydrochloride.

N-terminal amino acid sequence analysis of the two subunits, which were separated by SDS-PAGE and blotted to Immobilon polyvinylidene fluoride membranes, revealed two different sequences. For the α subunit, the following sequence was determined: 1-Met-Lys-Thr-Gln-Leu-Ser-Lys-Glu-Asp-Leu-Leu-Lys-Ala-Tyr-Arg-Lys-Met-Arg-Glu-Ile-Arg-Glu-Phe-Glu-Asp-Arg-Val-His-(Val)-Glu-30. For the β subunit, the following sequence was determined: 1-Ala-Arg-Lys-Ile-Met-Phe-Lys-Asp-Ala-Leu-Asn-Glu-Ala-Met-Arg-Leu-Glu-Met-Glu-Arg-Asp-Glu-Ser-(Val)-(Val)-Leu-Ile-Gly-Leu-29.

TABLE 4. Purification of Ao:DCPIP OR from *P. carbinolicus*

Step	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	39.5	29.2	1,153	3,286	2.85	1	100
Dialysis	64.5	16.7	1,077	3,741	3.47	1.22	114
DEAE-Sephacel	15.0	10.6	159.0	1,008	6.34	2.22	30.7
Triazine dyes	8.0	4.50	36.0	228	6.33	2.22	6.9
Mono Q HR	8.0	2.87	23.0	115	5.0	1.75	3.5

Reconstitution of the physiological overall reaction with purified Ao:DCPIP OR, DHLTA, and DHLDH. When purified Ao:DCPIP OR, DHLTA, and DHLDH from *P. carbinolicus* were combined in the presence of acetoin, NAD, and CoASH, formation of NADH could be measured photometrically (Table 5). Activity was strictly dependent on the presence of NAD, CoASH, and the enzymes Ao:DCPIP OR and DHLTA. A minor activity was detected in the absence of DHLDH; addition of TPP increased the activity 10-fold. With methylacetoin as a substrate, activity was fivefold higher than with acetoin. No activity was detected with diacetyl and 2-oxoglutarate as substrates, and very little activity (2% of acetoin-dependent activity) was found with pyruvate. No reduction of NADP occurred in the presence of acetoin and CoASH.

Induction of Ao:DCPIP OR, DHLTA, and DHLDH in strictly anaerobic, acetoin-utilizing bacteria. As shown previously, *P. carbinolicus* synthesizes Ao:DCPIP OR, DHLTA, and DHLDH during growth on acetoin (37). In cells that had been grown on ethylene glycol, these activities were absent. 2,3-Butanediol dehydrogenase was the only other enzyme detected which was present in acetoin-grown cells but was absent in ethylene glycol-grown cells (Table 6; 37). 2,3-Butanediol dehydrogenase functions probably as an additional sink for reducing equivalents during growth on acetoin and is responsible for the transient formation of 2,3-butanediol (14, 37). The formation of alcohol dehydrogenase, phosphotransacetylase, acetate kinase, and malate dehydro-

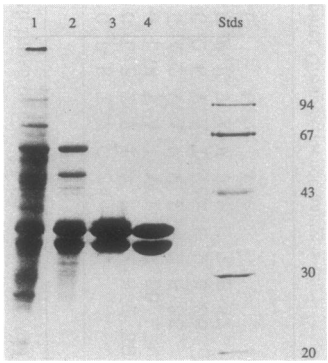

FIG. 3. SDS-PAGE of Ao:DCPIP OR from acetoin-grown *P. carbinolicus*. Samples were applied to 11.5% (wt/vol) SDS-polyacrylamide gels as described in Materials and Methods. Lanes: 1, 25 μg of protein from crude extract; 2, 10 μg of protein after chromatography of dialyzed crude extract on DEAE-Sephacel; 3, 10 μg of protein after chromatography on immobilized triazine dyes; 4, 10 μg of purified Ao:DCPIP OR after chromatography on Mono Q HR. Molecular masses of standards (Stds) are given on the right in kilodaltons. Protein was stained with Serva Blue R.

TABLE 5. Reconstitution of overall reaction from purified enzyme components, dependence on the presence of cofactors, and substrate specificity

Assay mixture	NAD reduction (μmol/min/ml)
Complete[a]	0.084
Without Ao:DCPIP OR	<0.001 (0.088)[b]
Without DHLTA	<0.001 (0.060)
Without DHLDH	0.003 (0.064)
Without CoA	<0.001 (0.036)
Without NAD	<0.001 (0.060)
Without TPP	0.008 (0.076)
Methylacetoin instead of acetoin	0.414
Pyruvate instead of acetoin	0.002
2-Oxoglutarate instead of acetoin	<0.001
Diacetyl instead of acetoin	<0.001
NADP instead of NAD	<0.001

[a] Contained, in 100 mM Tris hydrochloride (pH 7.5), 3.0 mM acetoin, 0.13 mM CoA, 2 mM NAD, 0.8 mM TPP, 0.5 mM $MgCl_2$, 0.07 mg of Ao:DCPIP OR per ml, 0.06 mg of DHLTA per ml, and 0.01 mg of DHLDH per ml. The reactions were started by addition of acetoin and measured at 30°C by monitoring A_{340}.

[b] Values in parentheses show the rate of NAD reduction after the omitted compound was added to the assay mixture.

genase did not vary significantly, regardless of whether the cells were grown on acetoin or on ethylene glycol (Table 6).

To our knowledge, *P. venetianus*, *P. acetylenicus*, *P. propionicus*, *C. magnum*, and *A. carbinolicum* are the only well-characterized strictly anaerobic, acetoin-utilizing bacteria other than *P. carbinolicus*. Depending on the major products of the fermentative catabolism of acetoin, these bacteria can be arranged in three physiological groups (16): (i) like *P. carbinolicus*, *P. venetianus* and *P. acetylenicus* disproportionate acetoin to acetate and ethanol; (ii) *P. propionicus* is able to carboxylate C_2 compounds by a reductive mechanism and forms acetate and propionate; and (iii) *C. magnum* and *A. carbinolicum* belong to the group of homoacetogenic bacteria.

These five bacteria were examined for the presence of Ao:DCPIP OR, DHLTA, and DHLDH during growth on acetoin or on one nonacetoinogenic substrate (Table 6). In addition, the activities of 2,3-butanediol dehydrogenase, acylating acetaldehyde dehydrogenase, alcohol dehydrogenase, phosphotransacetylase, acetate kinase, and malate dehydrogenase were measured.

Ao:DCPIP OR, DHLTA, and DHLDH were present at high activities only in acetoin-grown cells, whereas they were absent or scarcely detectable in cells grown on the nonacetoinogenic substrates (Table 6). In all strains, active Ao:DCPIP OR was detected exclusively in acetoin-grown cells. Even if low activities of DHLTA and DHLDH were detected in some strains grown on a nonacetoinogenic substrate, both enzymes were synthesized at severalfold-higher activities during growth on acetoin. These results clearly demonstrate that the formation of Ao:DCPIP OR, DHLTA, and DHLDH is induced during growth on acetoin in all strictly anaerobic bacteria examined in this study.

To analyze whether the differences in the specific activities of Ao:DCPIP OR, DHLTA, and DHLDH during growth on acetoin and on the nonacetoinogenic substrates resulted from protein synthesis or from activity control, the crude extracts from acetoin- and non-acetoin-grown cells of the different anaerobic acetoin-utilizing bacteria were examined by gel electrophoretic and immunological methods. In the protein pattern of crude extracts from acetoin-grown *P. carbinolicus*, Ao:DCPIP OR could be clearly identified as a

TABLE 6. Comparison of enzyme activities in crude extracts from acetoin-grown and non-acetoin-grown cells of strictly anaerobic bacteria[a]

Strain	Substrate	Sp act (U/mg)								
		Ao:DCPIP OR	DHLTA	DHLDH	2,3-Butanediol dehydrogenase	Acylating acetaldehyde dehydrogenase	Alcohol dehydrogenase	Phosphotrans-acetylase	Acetate kinase	Malate dehydrogenase
P. carbinolicus	Acetoin	2.9	19.0	2.5	0.12	0.027	0.24	17.5	15.0	0.15
	Ethylene glycol	<0.003	<0.02	<0.003	<0.003	0.017	0.22	19.5	10.5	0.33
P. venetianus	Acetoin	0.5	4.1	0.79	0.26	0.005	0.26	12.0	6.7	0.09
	Diethylene glycol	<0.003	<0.02	0.0045	0.008	0.025	0.14	26.9	9.4	0.07
P. acetylenicus	Acetoin	0.73	15.2	1.2	0.01	0.005	0.03	65.4	43.1	0.08
	Choline	<0.003	0.22	0.01	0.12	0.036	0.21	140.0	61.9	0.18
P. propionicus	Acetoin	0.10	8.5	1.3	0.05	<0.003	<0.003	7.1	1.5	43.9
	Lactate	<0.003	0.60	0.04	<0.003	ND[b]	2.8	7.7	0.8	19.7
A. carbinolicum	Acetoin	0.06	0.28	4.5	<0.003	<0.003	<0.003	0.09	0.25	0.06
	Ethanol	<0.003	0.05	<0.003	<0.003	ND	0.06	0.03	0.06	0.04
C. magnum	Acetoin	0.18	0.72	2.0	<0.003	0.42	0.05	4.1	2.5	0.07
	Glucose	<0.003	0.05	<0.003	<0.003	0.10	0.01	5.2	2.8	0.06

[a] The substrates were added from filter-sterilized stock solutions to the following final concentrations: acetoin, 0.3% (wt/vol); ethylene glycol, 0.3% (vol/vol); diethylene glycol, 0.3% (vol/vol); lactate, 0.3% (wt/vol); glucose, 0.3% (wt/vol); and ethanol, 0.15% (vol/vol). For growth of *A. carbinolicum*, the media were supplemented with 0.05% (wt/vol) yeast extract.

[b] ND, Not determined.

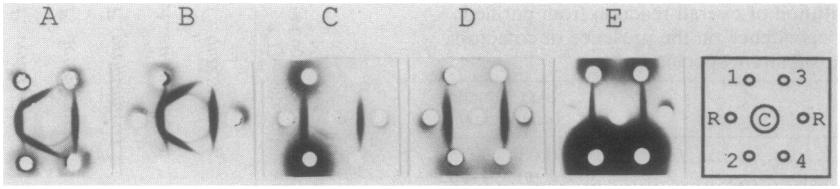

FIG. 4. Immunodiffusion test with crude extracts of strictly anaerobic acetoin-utilizing bacteria against anti-*P. carbinolicus* DHLDH IgGs. Wells: C, 85 μg of anti-*P. carbinolicus* DHLDH IgGs; R, acetoin-grown *P. carbinolicus*; A1 and A2, acetoin-grown *P. venetianus*; A3 and A4, diethylene glycol-grown *P. venetianus*; B1 and B2, acetoin-grown *P. acetylenicus*; B3 and B4, choline-grown *P. acetylenicus*; C1 and C2, acetoin-grown *P. propionicus*; C3 and C4, lactate-grown *P. propionicus*; D1 and D2, acetoin-grown *A. carbinolicum*; D3 and D4, ethanol-grown *A. carbinolicum*; E1 and E2, acetoin-grown *C. magnum*; E3 and E4, glucose-grown *C. magnum*. The protein content of the different crude extracts was 120 μg each in well R, ca. 300 μg each in wells 1 and 3, and ca. 600 μg each in wells 2 and 4.

dominant protein band by activity staining after nondenaturing PAGE, whereas this protein was absent in the protein pattern of ethylene glycol-grown cells (36). Analogous results were obtained with crude extracts of *P. venetianus* and *P. acetylenicus*. The protein patterns from acetoin- and from non-acetoin-grown cells of *P. propionicus*, *A. carbinolicum*, and *C. magnum* did not show such significant differences.

Similar results were obtained for DHLDH. On Western immunoblots from crude extracts of acetoin-grown *P. carbinolicus*, only one band was stained either by affinity staining or by immunostaining with anti-rabbit IgG alkaline phosphatase conjugate; this band was absent in ethylene glycol-grown cells. In double-immunodiffusion tests with anti-*P. carbinolicus* DHLDH IgGs, the crude extracts of acetoin-grown *P. venetianus* and *P. acetylenicus* gave strong cross-reactions which characterize the DHLDH from *P. carbinolicus*, *P. venetianus*, and *P. acetylenicus* as partially identical (Fig. 4). All other extracts showed no or barely detectable reactions. These strong similarities of the members of the acetoin-disproportionating group reflects their close phylogenetic relationship, as revealed from 16S rRNA sequence analysis (54). No cross-reaction was observed with crude extracts derived from acetoin-grown cells of *Alcaligenes eutrophus* H16, glucose-grown cells of *Escherichia coli* K-12, and glycine-grown cells of *Clostridium acidiurici*, *Clostridium sporogenes*, or *Clostridium sticklandii*.

For the third acetoin-specific protein, the DHLTA, it could be shown for *P. carbinolicus*, by comparison of crude extracts and purified DHLTA after electrophoresis in 4% (wt/vol) acrylamide gels, that this enzyme, in addition to Ao:DCPIP OR and DHLDH, is present only in acetoin-grown cells (Fig. 2B) and absent in ethylene glycol-grown cells. Anti-*P. carbinolicus* DHLTA IgGs reacted specifically with DHLTA, as revealed from immunostained Western blots of crude extracts derived from acetoin-grown cells of *P. carbinolicus*. Only one band was stained; this band was absent in crude extracts derived from ethylene glycol-grown cells. No immunological cross-reaction was observed in double immunodiffusion tests against crude extracts from *P. carbinolicus* or against crude extracts from the other anaerobic acetoin-utilizing bacteria.

DISCUSSION

Since the main fermentation products during growth on acetoin of all strictly anaerobic, acetoin-utilizing bacteria examined in this study are ethanol and/or acetate (or, in the case of *P. propionicus*, propionate derived from reductive carboxylated acetyl-CoA; 48) a C-C cleavage reaction must be catalyzed by these organisms during degradation of acetoin. For the catabolism of acetoin in *P. carbinolicus*, four possible pathways were discussed. Physiological and enzymatic investigations in addition to energetic considerations provided evidence that in *P. carbinolicus* acetoin is most likely cleaved into acetyl-CoA and acetaldehyde by an oxidative-thiolytic overall reaction (36). In crude extracts from acetoin-grown cells of *P. carbinolicus*, a CoASH- and NAD-dependent oxidative cleavage of acetoin into acetaldehyde and acetyl-CoA and of methylacetoin into acetone and acetyl-CoA has been demonstrated recently (37). In addition, it was shown that the formation of Ao:DCPIP OR, DHLTA, and DHLDH was induced during growth on acetoin or acetoinogenic substrates (37). These three enzymes were purified from crude extracts of *P. carbinolicus*; their characteristics show striking similarities to the enzyme components of 2-oxo acid dehydrogenase complexes of aerobic organisms.

Ao:DCPIP OR catalyzes the TPP-dependent cleavage of acetoin, methylacetoin, and diacetyl. The cleavage reaction of, for instance, acetoin may be analogous to the reaction that is catalyzed by the E_1 component of 2-oxo acid dehydrogenase complexes. Because of the different chemical structures of the substrates, the leaving group adjacent to the carbonyl carbon is a hydroxyethyl residue in the case of acetoin with Ao:DCPIP OR (resulting in the formation of acetaldehyde) or a carboxyl residue in the case of, for example, pyruvate with pyruvate dehydrogenase (resulting in the formation CO_2). The second product formed by the Ao:DCPIP-catalyzed cleavage reactions in the presence of the artificial electron acceptor DCPIP in vitro is acetate, which is probably hydrolytically split from acetyl-TPP after DCPIP-caused oxidation of hydroxyethyl-TPP (18). The in vitro formation of acetate in the presence of an artificial electron acceptor (i.e., ferricyanide or DCPIP) is well described for many different TPP-dependent enzymes, such as the E_1 components of 2-oxo acid dehydrogenase complexes (18, 24, 32, 43) and pyruvate decarboxylase (8) or glycolate with transketolase (27). Since no pyruvate- or 2-oxoglutarate-dependent reduction of DCPIP was measured with crude extracts and with purified Ao:DCPIP OR, it is unlikely that the cleavage of acetoin is catalyzed by nonspecific E_1 components of 2-oxo acid dehydrogenase complexes. The in vitro cleavage of diacetyl into stoichiometric amounts of acetate, which is catalyzed by Ao:DCPIP OR, seems to have no physiological relevance, as revealed from considerations of energy and from physiological experiments (36). In gas chromatographic analysis of the products of acetoin conversion in crude extracts in the presence of NAD and coenzyme A and with purified Ao:DCPIP OR in the presence of DCPIP, diacetyl could not be detected. Therefore, diacetyl is presumably neither the substrate for a physiological cleavage reaction nor a product of acetoin oxidation in *P. carbin-*

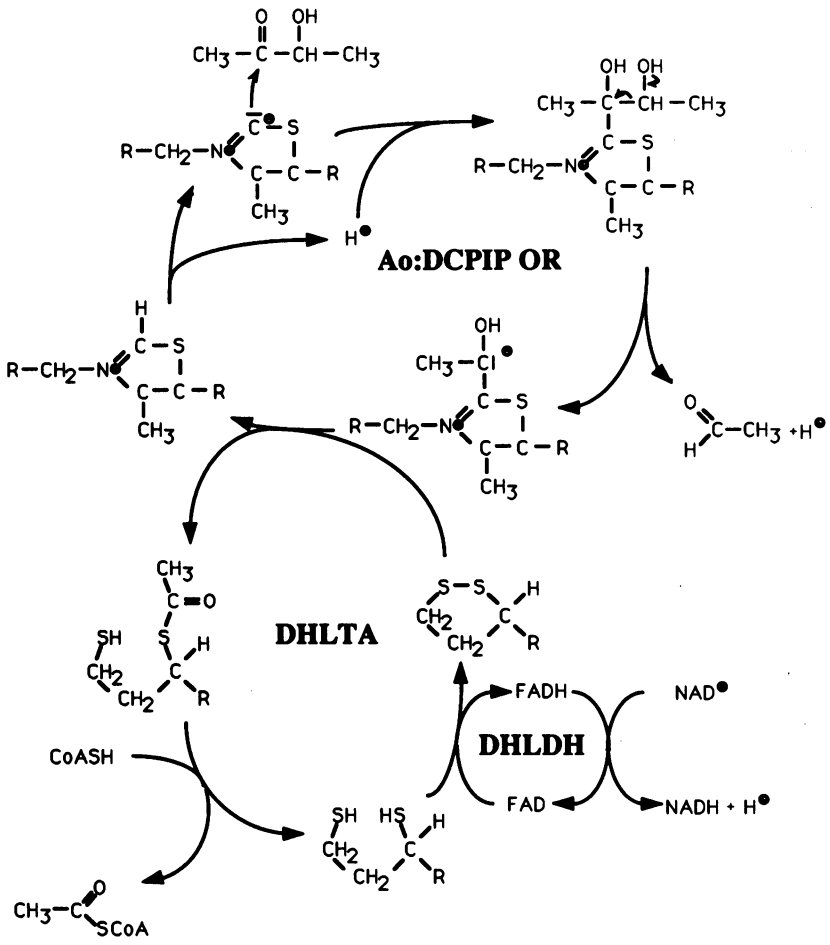

FIG. 5. Hypothetical cleavage of acetoin in *P. carbinolicus*.

olicus. From the molecular masses of the subunits and of the native Ao:DCPIP OR and from the identical intensities of the corresponding protein bands in SDS-gels after Coomassie staining, a tetrameric $\alpha_2\beta_2$ structure is most likely.

Purified DHLTA of *P. carbinolicus* shares a remarkable structural attribute with the E_2 components of 2-oxo acid dehydrogenases from other sources. The subunits of dihydrolipoamide acyltransferases from other organisms typically of one to three lipoyl-binding domain and one catalytic domain, which are connected through protease-sensitive hinge regions (5, 9, 20, 28, 40). Upon limited proteolysis under nondenaturing conditions, the hinge regions are cut and usually two fragments with similar molecular masses, representing the lipoyl-binding domains and the catalytic domain, were obtained. Activity is usually retained during proteolytic digestion, which can be explained by the finding that the acyl transfer from CoA to exogenous dihydrolipoamide is exclusively catalyzed by the catalytic subunit, regardless of whether the lipoyl-binding domain is connected. This rather unphysiological reaction is measured only in vitro and does not occur in vivo (6, 20, 28, 40). After time-limited tryptic digestion of DHLTA from *P. carbinolicus*, which was purified in the presence of protease inhibitors, two major fragments with M_rs of 28,500 and 32,500 and one minor fragment with an M_r of 52,500 were detected. No loss of enzyme activity occurred during the incubation period. The structural characteristic described above provides an explanation for the fact that fully active DHLTA, which was purified in the absence of protease inhibitors, exhibited an electrophoretic protein pattern resembling that of the tryptic digest obtained from controlled proteolysis of structurally intact DHLTA.

The NAD(H)-dependent DHLDH purified from acetoin-grown *P. carbinolicus* is a dimeric enzyme with identical subunits of M_r 54,000, each containing one noncovalently bound FAD. The N-terminal amino acid sequence of DHLDH, which includes the FAD-binding site, exhibits a high degree of homology to other DHLDHs. Therefore, the DHLDH of *P. carbinolicus* represents the classical and widespread type Ia of DHLDH (11). This type is usually involved as the E_3 component in aerobic 2-oxo acid dehydrogenase complexes (7, 41, 43, 53, 64); in addition, it is involved as the L or P_3 protein in the glycine cleavage systems of aerobic organisms (22, 61) and of the strict anaerobe *Peptostreptococcus glycinophilus* (3, 11, 26). Three minor groups (Ib, II, and III) of DHLDHs differ from type Ia in pyridine nucleotide specificity and in molecular mass; respectively, these enzymes are involved in the glycine degradation of strictly anaerobic, glycine-utilizing bacteria (11, 12, 17).

With purified Ao:DCPIP OR, DHLTA, and DHLDH from *P. carbinolicus* a TPP- and CoASH-dependent reduction of NAD in the presence of acetoin as well as of methylacetoin could be demonstrated. All three isolated enzymes were

required to constitute full activity. Only a minor activity or no activity was detected if acetoin was replaced by pyruvate or 2-oxoglutarate, respectively. It is therefore unlikely that the CoASH-dependent conversion of acetoin and methylacetoin is caused by an unspecific pyruvate or 2-oxoglutarate dehydrogenase complex. Since *P. carbinolicus* breaks down acetoin stoichiometrically to almost equal amounts of acetate and ethanol if acetoin is used as a carbon source and since the metabolism of this bacterium is strictly fermentative (45), the latter enzymes are not needed for catabolic purposes.

From the results of the reconstitution experiments and from the homologies of Ao:DCPIP OR, DHLTA, and DHLDH of *P. carbinolicus* to the E_1, E_2, and E_3 components of aerobic 2-oxo acid dehydrogenase complexes, it can be concluded that the CoASH- and NAD-dependent oxidative cleavage of acetoin is the key reaction of the anaerobic catabolism of acetoin in *P. carbinolicus*. This cleavage may be analogous to the overall reaction of 2-oxo acid dehydrogenase complexes (Fig. 5). The induction of Ao:DCPIP OR, DHLTA, and DHLDH during growth on acetoin in all of the anaerobic bacteria examined in this study indicates that a CoASH-dependent oxidative acetoin cleavage is a common step in the anaerobic degradation of acetoin. Further work must focus on the structural association of Ao:DCPIP OR, DHLTA, and DHLDH. Since Ao:DCPIP OR, DHLTA, and DHLDH can be easily separated during purification, the putative structural association is probably rather weak.

ACKNOWLEDGMENTS

This study was supported by grant Ste 386/3-1 from the Deutsche Forschungsgemeinschaft.

We thank H. Priefert and D. Dietrichs (both of the Institut für Mikrobiologie der Georg-August-Universität, Göttingen, Federal Republic of Germany) for providing crude cell extracts of *Alcaligenes eutrophus* H16, *Escherichia coli* K-12, *Clostridium acidiurici*, *C. sporogenes*, and *C. sticklandii*.

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