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Purification and Comparative Studies of Dihydrolipoamide Dehydrogenases from the Anaerobic, Glycine-Utilizing Bacteria *Peptostreptococcus glycinophilus*, *Clostridium cylindrosporum*, and *Clostridium sporogenes*

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Three different dihydrolipoamide dehydrogenases were purified to homogenity from the anaerobic glycine-utilizing bacteria *Clostridium cylindrosporum*, *Clostridium sporogenes*, and *Peptostreptococcus glycinophilus*, and their basic properties were determined. The enzyme isolated from *P. glycinophilus* showed the properties typical of dihydrolipoamide dehydrogenases: it was a dimer with a subunit molecular mass of 53,000 and contained 1 mol of flavin adenine dinucleotide and 2 redox-active sulfhydryl groups per subunit. Only NADH was active as a coenzyme for reduction of lipoamide. Spectra of the oxidized enzyme exhibited maxima at 230, 270, 353, and 453 nm, with shoulders at 370, 425, and 485 nm. The dihydrolipoamide dehydrogenases of *C. cylindrosporum* and *C. sporogenes* were very similar in their structural properties to the enzyme of *P. glycinophilus* except for their coenzyme specificity. The enzyme of *C. cylindrosporum* used NAD(H) as well as NADP(H), whereas the enzyme of *C. sporogenes* reacted only with NADP(H), and no reaction could be detected with NAD(H). Antibodies raised against the dihydrolipoamide dehydrogenase of *C. cylindrosporum* reacted with extracts of *Clostridium acidiurici*, *Clostridium purinolyticum*, and *Eubacterium angustum*, whereas antibodies raised against the enzymes of *P. glycinophilus* and *C. sporogenes* showed no cross-reaction with extracts from 42 organisms tested.

The enzyme dihydrolipoamide dehydrogenase belongs to the group of flavin-containing pyridine nucleotide disulfide oxidoreductases (7, 21, 50), like thioredoxin reductase (19), glutathione reductase (30), trypanothione reductase (34), bis-γ-glutamylcystine reductase (38), pantethine 4′,4″-diphosphate reductase (39), and mercuric reductase (15). The enzyme is known to be an integral component of the pyruvate, 2-oxoglutarate (7, 20, 21, 50), and branched-chain 2-oxoacid dehydrogenase (28, 36) and of the glycine decarboxylase complex (16, 17, 22, 35, 46). Glycine decarboxylase plays a major role in photorespiration, leading from 2 mol of phosphoglycolate to 1 mol of phosphoglycerate (46) and in glycine catabolism of anaerobic bacteria such as *Peptostreptococcus glycinophilus* (3, 14, 22), *Clostridium cylindrosporum* (13), and *Eubacterium acidaminophilum* (16–18, 51).

Dihydrolipoamide dehydrogenases have been isolated from various sources (6, 11, 20, 22, 33, 35, 36, 49). All dihydrolipoamide dehydrogenases are dimers consisting of two identical subunits, which contain 1 mol of flavin adenine dinucleotide (FAD) and two redox-active cysteine residues per subunit (21, 50). The molecular mass of the subunit was determined to be in the range of 49,000 to 58,000 by biochemical methods. However, sequence data indicate an M_r of 49,000 to 52,300 (5, 7, 37, 49). The enzymes are generally quite heat and proteolysis resistant and sensitive to some divalent cations (e.g., Hg^{2+}, Cu^{2+}) and to some arsenic derivatives (1, 21, 23, 33, 50). They react only with NAD(H) as a physiological coenzyme besides many artificial electron acceptors, but not with NADP(H) (6, 11, 22, 33, 35, 36, 49, 50).

Different NAD-dependent dihydrolipoamide dehydrogenases have been characterized, three from *Pseudomonas putida* and two from *Pseudomonas aeroginosa*, which show some structural and functional differences (6, 28, 35, 36). One enzyme is part of the 2-oxoglutarate dehydrogenase and probably of the pyruvate dehydrogenase complex (35); the second is part of the branched-chain 2-oxoacid dehydrogenase complex (36). During glycine oxidation by *P. putida*, only the dihydrolipoamide dehydrogenase of the 2-oxoglutarate dehydrogenase complex is involved, which has a subunit M_r of 56,000, but not the enzyme specific for valine with an M_r of 49,000 (35). A third NAD-dependent dihydrolipoamide dehydrogenase has been detected in mutants of *P. putida* with an intermediate M_r of 53,000 per subunit (6). From all these data, it seemed that the dihydrolipoamide dehydrogenases differ to some extent only in their molecular mass, but show considerable homologies in amino acid sequences (5–7, 37, 49).

So far, the recently described dihydrolipoamide dehydrogenase involved in the glycine decarboxylase complex of *Eubacterium acidaminophilum* is the only enzyme with deviating properties, such as its low molecular weight of 34,500 per subunit and the preference for NADP(H) as a coenzyme (17). With antibodies directed against this enzyme, cross-reactions were observed with extracts of *Clostridium cylindrosporum*, *Clostridium sporogenes*, and *Clostridium sticklandii* (17). Thus, it seemed reasonable to purify the dihydrolipoamide dehydrogenase from these glycine-utilizing anaerobes for comparative studies.

MATERIALS AND METHODS

Chemicals. Enzymes and coenzymes were obtained from Boehringer (Mannheim, Federal Republic of Germany [FRG]), fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) was from AHS/Deutschland GmbH (Munich, FRG), molecular weight markers, thio-

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NAD^+, and thio-$NADP^+$ were from Sigma (Deisenhofen, FRG), Sepharose CL-6B, DEAE-Sephacel, CNBr-activated Sepharose 4B, Sephadex G-200, Sepharose Q, and protein A-Sepharose CL-6B were from Pharmacia (Freiburg, FRG), DL-lipoamide and DL-lipoic acid were from Serva (Heidelberg, FRG), hydroxylapatite Biogel-HTP was from Bio-Rad Laboratories (Munich, FRG). DL-Dihydrolipoamide was prepared by reduction of DL-lipoamide with borohydride (17). All other chemicals were of the highest purity available from commercial sources.

Organisms and growth conditions. *C. cylindrosporum* (DSM 605) was anaerobically grown on 100 mM glycine and 12 mM uric acid as the substrate (2) supplemented with 0.1% (wt/vol) yeast extract; *C. sporogenes* (DSM 633) was anaerobically grown as described before (8); and *Peptostreptococcus glycinophilus* was grown anaerobically (14) with 50 mM glycine as the substrate supplemented with 0.1% (wt/vol) yeast extract. The cells were mass-cultured in 20-liter carboys at 37°C and harvested by centrifugation (8,000 × *g*) in the logarithmic growth phase. *C. acidiurici* (DSM 604), *C. purinolyticum* (DSM 1384), and *Eubacterium angustum* (DSM 1989) were grown on the medium described for *C. cylindrosporum*; *Acetobacteroides glycinophilus* (DSM 3078) and *Acidaminobacter hydrogenoformans* (DSM 2748) were grown on the medium described for *Eubacterium acidaminophilum* (51) supplemented with 0.2% (wt/vol) yeast extract; and *Peptostreptococcus barnesae* (DSM 3244) was grown as described before (32). Anaerobic cocci of Hare group I (NCTC 9801), Hare group III (NCTC 9803), Hare group IV (NCTC 9804), Hare group VIII (NCTC 9810), Hare group XI (NCTC 9811), *Peptostreptococcus magnus* (DSM 20472), *Peptostreptococcus micros* (DSM 20468), *Peptostreptococcus parvulus* (DSM 20469), *Peptostreptococcus prevotii* (DSM 20548), and *Peptostreptococcus variabilis* (ATCC 14955) were grown as described by Tziaka (Ph.D. thesis, University of Göttingen, Göttingen, FRG, 1987) on medium containing 50 mM glycine, 10 mM serine, 2.5 mM glucose, 2.5 mM fructose, and 1.2 mM uric acid, supplemented with 0.25% (wt/vol) yeast extract and 0.1% (vol/vol) Tween 80. *Peptococcus niger* (DSM 20475), *Peptostreptococcus anaerobius* (DSM 2949), *Peptostreptococcus asaccharolyticus* (DSM 29743), *Peptostreptococcus indolicus* (DSM 20464), *Peptostreptococcus productus* (DSM 2950), and *Peptostreptococcus tetradius* (DSM 2951) were grown on a complex medium as described by Tziaka (Ph.D. thesis, University of Göttingen, 1987) and the Deutsche Sammlung von Mikroorganismen (DSM) Catalogue of Strains (1986). Bacterium W6 was anaerobically grown on a freshwater medium containing 50 mM betaine, 10 mM glycine, and 25 mM alanine (C. Fendrich, Ph.D. thesis, University of Göttingen, 1988), and *Clostridium sticklandii* (DSM 519) was grown as described before (42). Media for *Acetobacterium woodii* (DSM 1030), *Alcaligenes eutrophus* H16 (DSM 428), *Arthrobacter globiformis* (DSM 20124), *Clostridium acetobutylicum* (DSM 792), *Clostridium cochlearium* (DSM 2153), *Escherichia coli* K-12 (DSM 498), *Eubacterium limosum* (DSM 20402), *Pseudomonas putida* (DSM 50202), *Spiromusa ovata* H1 (DSM 2662), *Azotobacter vinelandii* (DSM 2289), and *Bacillus subtilis* (DSM 401) are described in the Deutsche Sammlung von Mikroorganismen Catalogue of Strains.

Preparation of cell extract. Cells (wet weight) were suspended with 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM dithioerythritol, and 0.2 mM phenylmethylsulfonyl fluoride in a ratio of 1:1 (wt/vol) (*Peptostreptococcus glycinophilus*) or 1:2 (wt/vol) (*C. cylindrosporum* and *C. sporogenes*). After addition of 1 mg of lysozyme and 0.1 mg of DNase I per g of cells, the suspension was incubated at 37°C for 30 min. Cell suspensions of *C. cylindrosporum* and *C. sporogenes* were passed twice through a precooled French pressure cell at 147 MPa; cell suspensions of *Peptostreptococcus glycinophilus* were sonicated at 0°C for 15 times in 1-min intervals per 25 ml with intermittent cooling periods of 1 min. The cell debris was removed by centrifugation for 30 min at 12,000 × *g* at 4°C. The resulting supernatant (crude extract) was used for enzyme purification.

Enzyme assays. Dihydrolipoamide dehydrogenase was assayed spectrophotometrically at 30°C in (1-ml final volume) (i) 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM dithioerythritol, 0.4 mM NAD(P)H, and 1.5 mM lipoamide or (ii) 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM dithioerythritol, 0.4 mM $NAD(P)^+$, and 1.5 mM dihydrolipoamide. The reaction was started with lipoamide (i) or dihydrolipoamide (ii), and the change in A_{365} (i) or A_{340} (ii) was determined over time. Stock solutions (100 mM) of lipoamide and dihydrolipoamide were dissolved in dimethyl sulfoxide and stored at −20°C. Electron transferase activity was assayed at 30°C in 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 0.4 mM NAD(P)H, and different concentrations of various electron acceptors (41, 50). In a final volume of 1 ml, the reaction was started by the addition of enzyme or electron acceptor, and the change in absorbance of the correspondent electron acceptor was determined over time. Transhydrogenase activity (41) was assayed spectrophotometrically at 30°C in a final volume of 1 ml in 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM dithioerythritol, 0.4 mM NAD(P)H, and 0.4 mM thio-$NAD(P)^+$. The reaction was started with NAD(P)H, and the change in A_{395} was determined over time. Specific activity is expressed as micromoles of NAD(P)H consumed or produced per minute per milligram of protein at 30°C.

Dihydrolipoamide dehydrogenase activity was detected in polyacrylamide gels with (i) an anaerobically kept reaction mixture containing 5 mM benzyl viologen, 2 mM dihydrolipoamide, 1 mM EDTA, and 1 mM dithioerythritol in 50 mM potassium phosphate buffer, pH 7.8, or (ii) a reaction mixture containing 2 mM lipoamide, 0.4 mM NAD(P)H, 0.5 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and 1 mM EDTA in 50 mM potassium phosphate buffer, pH 7.8. The gel was incubated in the reaction mixture at 37°C until violet (i) or yellow (ii) bands appeared. Transfer of the proteins onto nitrocellulose was still possible after this procedure.

Kinetic studies were carried out at the pH optimum in 1-ml cuvettes containing (i) 50 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA and 1 mM dithioerythritol [for K_m determination of NAD(P)H and lipoamide] or (ii) 50 mM TAPS (*N*-tris[hydroxymethyl]-methyl-3-amino-propanesulfonic acid) buffer, pH 9.0, with 1 mM EDTA and 1 mM dithioerythritol [for K_m determination of $NAD(P)^+$ and dihydrolipoamide]. NAD(P)H or $NAD(P)^+$ concentration was varied from 0.05 to 4.0 mM at 5 mM lipoamide or dihydrolipoamide, respectively, and lipoamide or dihydrolipoamide concentration was varied from 0.5 to 4 mM at 1 mM NAD(P)H or $NAD(P)^+$, respectively. The dihydrolipoamide dehydrogenase concentration was 1 to 5 μg of enzyme per assay.

Purification of dihydrolipoamide dehydrogenase. To purify the dihydrolipoamide dehydrogenase of *Peptostreptococcus glycinophilus*, ammonium sulfate was added to 300 ml of

crude extract (6,525 mg of protein) to give 55% saturation. The solution was stirred for 15 min at 4°C. The precipitate was removed by centrifugation for 30 min at 24,000 × *g*, and the supernatant was brought to 100% ammonium sulfate saturation. After stirring for 15 min at 4°C and centrifugation for 30 min at 24,000 × *g*, the precipitate was suspended in buffer A (50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithioerythritol) and dialyzed for 18 h against buffer A at 4°C. This pool was applied to a column (2.6 by 11 cm; bed volume, 60 ml) of DEAE-Sephacel previously equilibrated with buffer A. After the column was washed with 200 ml of this buffer, proteins were eluted with a gradient of 0 to 1 M KCl in 500 ml of buffer A at 4°C and a flow rate of 30 ml/h. Dihydrolipoamide dehydrogenase was eluted at approximately 0.25 M KCl. Fractions containing activity were pooled, dialyzed for 15 h against buffer B (50 mM Tris buffer, pH 8.2, containing 1 mM EDTA and 1 mM dithioerythritol), and applied to a column (1.6 by 10 cm; bed volume, 20 ml) of Sepharose Q previously equilibrated with buffer B. After the column was washed with 40 ml of this buffer, proteins were eluted by a gradient of 0 to 1 M KCl in 400 ml of buffer B at a flow rate of 15 ml/h. Dihydrolipoamide dehydrogenase was eluted at approximately 0.45 M KCl. The pooled enzyme solution was dialyzed for 15 h against buffer C (10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM dithioerythritol) and applied to a hydroxylapatite Bio-Gel HTP column (1.5 by 8 cm; bed volume, 14 ml) previously equilibrated with buffer C. After the column was washed with 40 ml of this buffer, the proteins were eluted with a gradient of 0.01 to 0.5 M potassium phosphate in 200 ml of buffer C at 4°C and a flow rate of 15 ml/h. Dihydrolipoamide dehydrogenase activity eluted at approximately 0.21 M potassium phosphate. Fractions with dihydrolipoamide dehydrogenase activity were pooled and concentrated by ultrafiltration (Ultrafilter SM 14549; Sartorius, Göttingen, FRG) to a final volume of 2.3 ml. The last step consisted of preparative polyacrylamide gel electrophoresis with an 8% polyacrylamide gel, pH 8.6 (14 by 14 by 0.6 cm). The enzyme solution was mixed with saccharose crystals and 400 μl of 0.05% bromophenol blue and applied to the gel surface. Electrophoresis was carried out at 200 V for 8 h at 5 mM Tris–38 mM glycine buffer, pH 8.6, at 4°C. After electrophoresis, a yellow fluorescent flavin band could be detected in the gel, which correlated with dihydrolipoamide dehydrogenase activity. The gel containing this yellow band was cut out, and the protein was eluted by electroelution with a Biotrap BT 1000 (Schleicher & Schüll, Dassel, FRG) filled with 5 mM Tris–38 mM glycine buffer, pH 8.6, at 200 V for 12 h at 4°C. The eluted yellow enzyme solution was dialyzed against buffer A for 15 h at 4°C and stored at −20°C.

The purification of the dihydrolipoamide dehydrogenase of *C. cylindrosporum* was carried out by the same procedure as described for *Peptostreptococcus glycinophilus* except that the purification was started with 40 ml of crude extract (1,497 mg of protein) which was applied to the DEAE-Sephacel column without a preceding ammonium sulfate precipitation.

The purification of the dihydrolipoamide dehydrogenase of *C. sporogenes* was carried out in the same manner as described for *C. cylindrosporum* except that the purification was started with 200 ml of crude extract (2,880 mg of protein) which was applied to the DEAE-Sephacel column. After preparative gel electrophoresis, two chromatographies on Mono Q HR 5/5 columns (0.5 by 5 cm) at different pHs (pH 7.8 and 6.8, respectively) were carried out to remove a contaminating protein which exhibited an M_r of about 60,000 in sodium dodecyl sulfate (SDS) gels. Dihydrolipoamide dehydrogenase eluted at approximately 0.30 M KCl in both cases.

After specific antibodies against the three different dihydrolipoamide dehydrogenases became available, the enzymes were purified by immunoabsorption and subsequent electroelution as described (17). To obtain homogeneity, the enzyme solution was separated on a Mono Q HR 5/5 column equilibrated with 50 mM Tris buffer containing 1 mM EDTA and 1 mM dithioerythritol, pH 8.2, and a gradient of 0 to 1 M KCl in 50 ml of the same buffer.

Electrophoresis. Fractions containing enzyme activity were tested for homogeneity by SDS-polyacrylamide gel electrophoresis (25). Native polyacrylamide gel electrophoresis under nondenaturing conditions with 8% polyacrylamide gels was performed as described in Technical Bulletin MKR-137 (Sigma Chemical Co., St. Louis, Mo.). Linear polyacrylamide gradient gels (27) were performed with a polyacrylamide gradient from 4 to 28%. Electrophoresis of these gels was carried out at 100 V for 15 h with 5 mM Tris–38 mM glycine buffer, pH 8.6, at 4°C. The gels were stained either with Coomassie Blue (47) or with a silver stain kit (Biometra GmbH, Göttingen, FRG) according to the instructions of the manufacturer.

Molecular weight determinations. The molecular weight was determined by using SDS-polyacrylamide gel electrophoresis and Sigma molecular weight marker kit MW-SDS-70L. The native molecular weight was determined by gel permeation chromatography on Superose 12 (Pharmacia, Freiburg, FRG) (2.6 by 60 cm; bed volume, 115 ml) with bovine liver catalase (M_r 240,000), rabbit muscle aldolase (M_r 158,000), bovine serum albumin (M_r 66,000), and bovine pancreas chymotrypsinogen (M_r 25,000). Molecular mass was further determined in native polyacrylamide gel electrophoresis with different concentrations of acrylamide as described in Technical Bulletin MKR-137. Native polyacrylamide gel gradients (27) were formed from 4 to 28% by using horse spleen ferritin (M_r 450,000), bovine liver catalase (M_r 240,000), bovine serum albumin (M_r 66,000), and egg albumin (M_r 45,000).

Thiol titration. The number of DTNB-titrable thiols in oxidized and NAD(P)H-reduced protein was determined under denaturing conditions as described before (34). The reaction mixture contained 3.75 mM dihydrolipoamide dehydrogenase, 0.1 M Tris (pH 8.6), 1.0 mM EDTA, and 6.0 M guanidine hydrochloride. After 10 min, DTNB was added to a final concentration of 0.1 mM, and the increase in A_{412} was measured. For monitoring the thiol groups in the reduced enzyme, the protein was treated with 0.3 mM NAD(P)H for 1 h before denaturation. For stoichiometric determinations, an extinction coefficient ε_{412} of 13.6 mM^{-1} cm^{-1} was used.

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*. The supernatant was analyzed by thin-layer chromatography (TLC) on TLC-cellulose plates (20 by 20 cm; Merck, Darmstadt, FRG) with *n*-butanol–acetic acid–water (4:3:3) as the solvent with FAD, flavin mononucleotide, and riboflavin as references. For stoichiometric determinations, an extinction coefficient ε_{450} of 11.3 mM^{-1} cm^{-1} was used.

Determination of protein and protein spectra. Protein was determined by the method of Bradford (4). Solutions of bovine serum albumin in water were used for standards. Protein spectra were obtained as described before (17).

Immunological techniques. Antisera were raised in rabbits

TABLE 1. Coenzyme specificity of dihydrolipoamide dehydrogenase activity in cell extracts of different bacteria

Organism	Sp act (U/mg)	
	NADH	NADPH
Acetobacteroides glycinophilus	2.2	ND[a]
Azotobacter vinelandii	1.3	ND
Bacillus subtilis	0.16	ND
Eubacterium limosum	1.1	ND
Peptostreptococcus glycinophilus	0.95	ND
Clostridium acidiurici	5.7	9.7
Clostridium cylindrosporum	4.5	7.1
Clostridium purinolyticum	1.3	1.7
Eubacterium angustum	0.05	0.07
Peptostreptococcus barnesae	0.11	0.12
Peptostreptococcus H IX 9811[b]	0.92	0.98
Peptostreptococcus H IX 9821	0.33	0.33
Peptostreptococcus micros	0.30	0.32
Bacterium W6	0.01	0.09
Clostridium sporogenes	ND	0.10
Eubacterium acidaminophilum	0.01	0.10
Peptrostreptococcus H III 9803	ND	0.04
Peptostreptococcus H IV 9804	ND	0.08
Peptostreptococcus parvulus	ND	0.21
Peptostreptococcus variabilis	0.01	0.17

[a] ND, No enzyme activity detectable.
[b] Hare group (H) and NCTC strain number.

(2.5 kg, 6 months old) against the purified dihydrolipoamide dehydrogenases by intramuscular injection of 200 to 300 μg of protein together with complete Freund adjuvant. A subcutaneous booster injection with 100 to 200 μg of protein together with incomplete Freund adjuvant was given 2 to 3 weeks later. Another 2 weeks later, the rabbits were bled. Purification of IgGs from the resulting serum was carried out on protein A-Sepharose CL-6B (24). The determination of antibody titer against dihydrolipoamide dehydrogenases was determined by double immunodiffusion tests (29), the specificity of antibodies raised against the enzymes was demonstrated by double immunofluorescence labeling of blotted proteins (40), and quantitation of dihydrolipoamide dehydrogenase in extracts was done by rocket immunoelectrophoresis (26, 48). With preimmune serum taken before immunization procedures, no cross-reactions could be detected. For analysis of relationships, extracts from several other bacteria were tested in double immunodiffusion tests, rocket immunoelectrophoresis, and the Western blot technique as described before (17, 24).

RESULTS

Extracts were screened for an NADP-dependent dihydrolipoamide dehydrogenase activity, and it was detected in several anaerobic bacteria (Table 1). *C. cylindrosporum* and *C. sporogenes* were chosen for further studies because their extracts reacted with antibodies raised against the dihydrolipoamide dehydrogenase of *Eubacterium acidaminophilum* (17). *Peptostreptococcus glycinophilus* was included for comparison to look for possible isoenzymes and to extend earlier studies (3, 22).

Purification of dihydrolipoamide dehydrogenases. After cell breakage and removal of cell debris, about 90% of dihydrolipoamide dehydrogenase activity was found in the supernatant of the three organisms tested. The dihydrolipoamide dehydrogenase of *Peptostreptococcus glycinophilus* was obtained with 233-fold-increased specific activity and 12% recovery (Table 2), and the purification of the dihydrolipoamide dehydrogenase of *C. cylindrosporum* resulted in a yellow enzyme solution with 68-fold-increased specific activity and 14% recovery (Table 2). The purification of the dihydrolipoamide dehydrogenase of *C. sporogenes* was done similarly to that of the *C. cylindrosporum* enzyme. Contaminating proteins were finally separated by chromatography

TABLE 2. Purification of dihydrolipoamide dehydrogenases from *Peptostreptococcus glycinophilus*, *C. cylindrosporum*, and *C. sporogenes*

Organism and step	Volume (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Peptostreptococcus glycinophilus						
Crude extract	300	6,000	6,525	0.92	1	100
$(NH_4)_2SO_4$	90	3,296	1,110	3.0	3.2	55
DEAE-Sephacel	33	2,463	277	8.9	9.7	41
Sepharose Q	25	2,226	258	39	42	37
Hydroxylapatite	2.3	1,387	8.8	158	172	23
Prep.PAGE[a]	2.0	727	3.4	214	233	12
C. cylindrosporum						
Crude extract	41	10,619	1,497	7.1	1	100
DEAE-Sephacel	29	9,889	470	21.1	3.1	93
Sepharose Q	14	6,523	127	51	7.2	61
Hydroxylapatite	1.6	2,860	18	160	22	27
Prep.PAGE	2.9	1,532	3.2	483	68	14
C. sporogenes						
Crude extract	200	114	2,880	0.04	1	100
DEAE-Sephacel	130	203	975	0.11	2.6	90
Sepharose Q	35	94	238	0.39	9.9	82
Hydroxylapatite	33	39	10.6	3.7	93	34
Prep.PAGE	1.8	29	2.4	17	416	25
Mono Q HR	1.0	15.3	0.4	36	910	13

[a] Prep.PAGE, Preparative polyacrylamide gel electrophoresis.

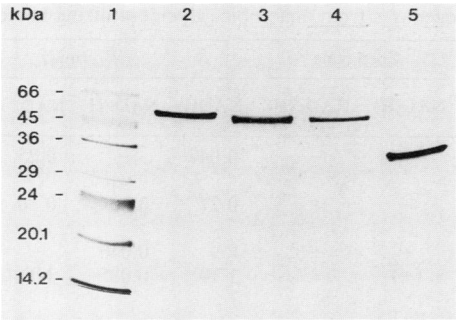

FIG. 1. Polyacrylamide gel electrophoresis of dihydrolipoamide dehydrogenases. The proteins were separated by SDS-polyacrylamide gel electrophoresis (14%) and determined by silver staining. Lane 1, 10 μg of marker proteins; lanes 2 to 5, 2 μg of dihydrolipoamide dehydrogenase from *Peptostreptococcus glycinophilus*, *C. cylindrosporum*, *C. sporogenes*, and *Eubacterium acidaminophilum*, respectively.

on Mono Q HR. The enzyme exhibited 910-fold-increased specific activity and 13% recovery (Table 2).

Both SDS-polyacrylamide and native linear polyacrylamide gradient gel electrophoresis revealed only a single protein band in each case (Fig. 1).

By using the different anti-dihydrolipoamide dehydrogenase IgG Sepharose 4B columns and subsequent electroelution, the three enzymes could be obtained in high purity in one step. By chromatography on a Mono Q HR 5/5 column, contaminating protein were removed.

The enzymes were stored at −20°C. The dihydrolipoamide dehydrogenase of *C. cylindrosporum* lost about 60% of its specific activity within 6 months, whereas the enzymes of *Peptostreptococcus glycinophilus* and *C. sporogenes* retained about 90% of their specific activity after this time.

Molecular weight. A subunit M_r of about 53,000 was calculated for the enzyme of *Peptostreptococcus glycinophilus* and an M_r of about 52,000 was estimated for the enzymes of *C. cylindrosporum* and *C. sporogenes* by SDS-polyacrylamide gel electrophoresis and comparison of the electrophoretic mobility with standard proteins. Molecular weight estimations for the native enzymes by gel filtration

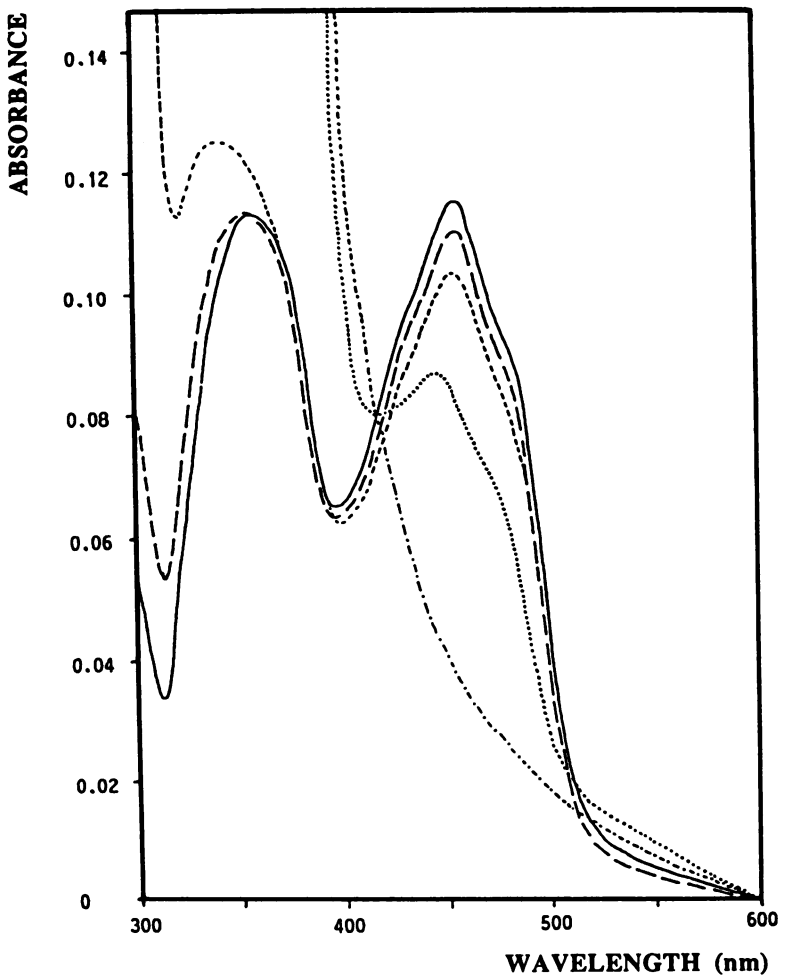

FIG. 2. Absorption spectra of the dihydrolipoamide dehydrogenase of *C. cylindrosporum*. Spectra were taken under an N_2 atmosphere. The enzyme solution (1.1 mg/ml) was dialyzed against an anaerobic 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA. The oxidized protein (——) was reduced by NAD(P)H to an FAD ratio of 1:1 (– – –), 10:1 (------), 100:1 (· · · · · ·), and a molar excess of dithionite (- · - · - · -).

TABLE 3. Structural and functional properties of dihydrolipoamide dehydrogenases from anaerobic, glycine-utilizing bacteria

Enzyme class	Representative organism	Structure	Mol wt		FAD content/ subunit	$E_{vis/max}$ (nm)	Coenzyme		K_m (mM)			
			Native	Subunit			NAD(H)	NADP(H)	NADH	NADPH	NAD^+	$NADP^+$
Ia	*Peptostreptococcus glycinophilus*	Dimer	105,000	53,000	1.08	453	+	−	0.047	−	0.25	−
Ib	*C. cylindrosporum*	Dimer	105,000	52,000	0.85	453	+	+	0.27	0.20	0.50	0.83
Ic	*C. sporogenes*	Dimer	100,000	52,000	0.88	454	−	+	−	0.040	−	0.022
II	*Eubacterium acidaminophilum*[f]	Dimer	68,000	34,500	1.07	453	(+)	+	1.10	0.085	ND	ND

[a] $LipS_2$, Lipoamide; $Lip(SH)_2$, dihydrolipoamide.
[b] Tested with NAD(P)H and lipoamide.
[c] Valid for both coenzymes.
[d] NADPH was used.
[e] ND, Not determined.
[f] Data are from reference 17.

chromatography with standard proteins resulted in an M_r of about 105,000 for the *Peptostreptococcus glycinophilus* and *C. cylindrosporum* enzymes and an M_r of about 100,000 for the *C. sporogenes* enzyme. Data obtained by native polyacrylamide gel electrophoresis and native polyacrylamide gel gradients both resulted in a lower M_r of about 88,000 for the *Peptostreptococcus glycinophilus* and *C. cylindrosporum* enzymes and of about 86,000 for the *C. sporogenes* enzyme. From these data, we still conclude that these dihydrolipoamide dehydrogenases consist of two identical subunits and that the M_r of the native enzymes is about 100,000, like that of other dihydrolipoamide dehydrogenases (6, 7, 11, 33, 37, 50), for the differences in native molecular mass determination might be based on a high positive net charge of these enzymes.

Spectra and FAD content. Spectra of these three dihydrolipoamide dehydrogenases showed absorption maxima at 230, 270, 353, and 453 nm, with shoulders at 370, 425, and 485 nm, which is typical of these flavoproteins (21, 50).

After reducing the enzymes with NADH (*Peptostreptococcus glycinophilus*), NAD(P)H (*C. cylindrosporum*), or NADPH (*C. sporogenes*), the maxima at 453 nm were reduced and shifted to shorter wavelengths. However, a high excess of reduction equivalents was necessary to reduce the flavin component in all three dihydrolipoamide dehydrogenases. An NAD(P)H-to-FAD ratio of 100 resulted in an absorption decrease at 453 nm of about 50% for the dihydrolipoamide dehydrogenase from *C. cylindrosporum* (Fig. 2). A molar excess of dithionite resulted in a complete absorption decrease at 453 nm. The A_{280}-A_{450} ratio of the native enzyme was 5.7 for *Peptostreptococcus glycinophilus*, 5.0 for *C. cylindrosporum*, and 5.2 for *C. sporogenes*. After liberation of the flavin moiety by thermal denaturation, the flavin component of all three dihydrolipoamide dehydrogenases was identified as FAD by thin-layer chromatography. The FAD content was calculated to be, per subunit, 1.08 mol for *Peptostreptococcus glycinophilus*, 0.85 mol for *C. cylindrosporum*, and 0.88 mol for *C. sporogenes*. From these data, we conclude that the three dihydrolipoamide dehydrogenases contain 1 mol of FAD per subunit.

Involvement of a redox-active disulfide. The effect of divalent cations on the catalytic activity was determined by incubating the enzymes (1 μg) for 30 min at room temperature with 0.1 mM of the divalent cation with chloride as the counterion in 50 mM potassium phosphate buffer, pH 7.8, in the presence 0.4 mM NAD(P)H, and by starting the reaction with lipoamide. The dihydrolipoamide dehydrogenase activities of *Peptostreptococcus glycinophilus*, *C. cylindrosporum*, and *C. sporogenes* were completely inhibited by Cu^{2+}, Cd^{2+}, and Zn^{2+} and, in decreasing order, by Co^{2+}, Pb^{2+}, and Ni^{2+}. In the absence of dithioerythritol, a spontaneous reduction of Hg^{2+} to Hg^+ by NAD(P)H prevented a study of the Hg^{2+} influence in this test system. Therefore, dihydrolipoamide dehydrogenase (1 μg) was incubated with 0.1 mM Hg^{2+} in the absence of dithioerythritol for 30 min at room temperature, followed by addition of 1 mM dithioerythritol to bind free Hg^{2+} and by starting with lipoamide. All three enzymes were completely inhibited by Hg^{2+}. Interestingly, the dihydrolipoamide dehydrogenase of *Eubacterium acidaminophilum* exhibits a high resistance against Zn^{2+} and Hg^{2+} (17). The same inhibitory effects by divalent cations were obtained for the reverse reaction starting with dihydrolipoamide. Arsenite and phenylarsinoxide, but not arsenate, react with vicinal thiol groups (1, 23). Their influence on the catalytic activity was determined by a 30-min incubation of the NAD(P)H-reduced (0.4 mM) enzymes at room temperature with 1 mM arsenite, arsenate, or phenylarsinoxide in 50 mM potassium phosphate buffer, pH 7.8, and starting with lipoamide. The enzyme activities of all three organisms were completely inhibited by treatment with 1 mM arsenite or phenylarsinoxide, but no significant loss in enzyme activity was observed with 1 mM arsenate.

Both oxidized and NAD(P)H-reduced enzyme was denatured with guanidine hydrochloride and reacted with DTNB. The difference in thiol content was found to be 1.6 thiols in the case of *Peptostreptococcus glycinophilus* and 1.7 thiols in the case of *C. cylindrosporum*. From these data, we conclude that a redox-active disulfide is involved at the catalytic active site of the enzymes.

Substrate specificity and kinetic properties. The purified dihydrolipoamide dehydrogenase of *Peptostreptococcus glycinophilus* was highly specific for NAD(H) as a coenzyme. No reaction was observed with NADP(H). The enzyme of *C. cylindrosporum* was found to use NADP(H) as well as NAD(H), both with high specific activities and about the same K_m for the pyridine nucleotides (Tables 1 and 3). The enzyme of *C. sporogenes* reacted only with NADP(H), representing a new type.

One of the characteristics of dihydrolipoamide dehydrogenases is the variety of electron acceptors to be used (41, 50). The enzymes of *C. cylindrosporum*, *C. sporogenes*, and *Peptostreptococcus glycinophilus* could use benzyl viologen, dichloroindophenol, ferricyanid, lipoamide, lipoic acid, menadione, methylene blue, and Nitro Blue Tetrazolium as

TABLE 3—*Continued*

pH optima[a]		Highest sp act[b] (U/mg)	Enzyme content in cell extract (%)	Immunological relationship to protein of:	Structural + biochemical relationship to corresponding enzyme	Common properties of organisms
NAD(P)H + $LipS_2$	$NAD(P)^+$ + $Lip(SH)_2$					
7.0	9.0	214	0.38	–	Only previously known type	
7.0[c]	9.0[c]	483[d]	0.50	*C. acidiurici*, *C. purinolyticum*, *Eubacterium angustum*	Hare group IX, *P. barnesae*, *P. micros*	Uric acid and glycine utilization
7.0	8.5–8.8	86	ND[e]	–	Bacterium W6	Stickland reactions
ND	ND	9[d]	1.4	Bacterium W6, *C. cylindrosporum*, *C. sporogenes*, *C. sticklandii*		Stickland reactions

an electron acceptor with the respective reduced pyridine nucleotide as the electron donor. A weak reduction of oxygen was observed under aerobic conditions.

Another characteristic of dihydrolipoamide dehydrogenases is a transhydrogenase activity (41). The enzyme of *Peptostreptococcus glycinophilus* transferred electrons from NADH to thio-NAD^+ (6.0 U/mg) but not to thio-$NADP^+$, that of *C. sporogenes* transferred electrons from NADPH to thio-$NADP^+$ (2.8 U/mg) but not to thio-NAD^+, and the enzyme of *C. cylindrosporum* was able to transfer electrons from both NADPH and NADH to both thio-$NADP^+$ (10.1 and 10.7 U/mg, respectively) and thio-NAD^+ (5.2 and 5.5 U/mg, respectively). Thus, the transhydrogenase activity correlated well with the coenzyme specificity observed before.

Immunological studies. The specificity of the isolated antibodies against the enzymes was proved by the Western immunoblot technique. Only one fluorescent band with a subunit M_r of about 53,000 (*Peptostreptococcus glycinophilus*) or 52,000 (*C. cylindrosporum* and *C. sporogenes*) was detected after SDS-polyacrylamide gel electrophoresis in extracts of the respective organisms. Extracts from various anaerobic and aerobic organisms as listed under Materials and Methods, most of them glycine- or purine-utilizing bacteria, were tested for cross-reactivity in double immunodiffusion tests and by rocket immunoelectrophoresis. With anti-*Peptostreptococcus glycinophilus* dihydrolipoamide dehydrogenase IgGs and anti-*C. sporogenes* dihydrolipoamide dehydrogenase IgGs, no cross-reactions could be detected with other organisms. However, anti-*C. cylindrosporum* dihydrolipoamide dehydrogenase IgGs reacted with extracts of *C. acidiurici*, *C. purinolyticum*, and *Eubacterium angustum*. Extracts of these latter organisms separated by linear polyacrylamide gradient gel electrophoresis were subjected to an activity stain. The stain with DTNB resulted in a single stained band with an M_r of about 88,000. With benzyl viologen as the electron acceptor, the polyacrylamide gels showed two stained bands with an M_r of about 88,000 and about 63,000. Only the more intensely stained bands with the higher M_r reacted with anti-*C. cylindrosporum* dihydrolipoamide dehydrogenase IgGs, as shown by double immunofluorescence labeling after blotting the proteins onto nitrocellulose filters, whereas the band with the lower M_r reacted with corresponding antibodies raised against the enzyme of *Eubacterium acidaminophilum* in the case of *C. cylindrosporum* and *C. acidiurici*.

DISCUSSION

This study was initiated by the discovery of an atypically small NADP-preferring dihydrolipoamide dehydrogenase which was found in the anaerobic, glycine-utilizing *Eubacterium acidaminophilum* (17). In this organism, this enzyme is involved in both the glycine decarboxylase and glycine reductase complex (18). Other strictly anaerobic, glycine-utilizing bacteria were examined first. Our screening established that many anaerobic organisms contained dihydrolipoamide dehydrogenase activities with NADP(H) as the coenzyme, using it either exclusively or besides NAD(H). According to this study, dihydrolipoamide dehydrogenases can be classified by their pyridine nucleotide coenzyme specificity and molecular mass into four different types (Table 3).

The dihydrolipoamide dehydrogenase isolated from *Peptostreptococcus glycinophilus* represented the classical type of this enzyme (type Ia in Table 3), having an M_r of 53,000 per subunit and reacting only with NAD(H). *Peptostreptococcus glycinophilus*, like *Eubacterium acidaminophilum*, has both an active glycine decarboxylase and glycine reductase multienzyme complex and thus is able to grow on glycine as the sole carbon and energy source (3, 14, 22). *Peptostreptococcus glycinophilus* metabolizes glycine preferentially via the glycine reductase complex, which requires selenium (14). Most of the glycine decarboxylase proteins of *Peptostreptococcus glycinophilus* have been characterized (3, 22). The dihydrolipoamide dehydrogenase isolated by us seems to be the same enzyme as studied before (3, 22), although the FAD content was rather low in a former study. We now also checked this organism for another dihydrolipoamide dehydrogenase (i.e., with properties similar to those of the enzyme isolated from *Eubacterium acidaminophilum*). However, only one enzyme activity was detected by various means, including labeling the cells with [^{14}C]riboflavin as done previously (17). The methylenetetrahydrofolate dehydrogenase, the second enzyme involved in the oxidation of glycine to CO_2, was found to be $NADP^+$ specific (31), whereas the coenzyme specificity of the formate dehydrogenase has not been studied so far. Thus, *Peptostreptococcus glycinophilus* should contain a protein with transhydrogenase activity or should be able to regenerate both coenzymes in the glycine reductase reaction. Due to its instability, the latter activity could only be deducted from labeling studies (14).

The dihydrolipoamide dehydrogenase of *C. cylindrosporum* constituted a new type of dihydrolipoamide dehydrogenase (type Ib in Table 3) in reacting with both NADP(H) and NAD(H) with high specific activities, but exhibiting the classical structure of two identical subunits with a subunit M_r of about 52,000. *C. cylindrosporum* is an obligately purine-utilizing anaerobe (2, 13). Purine is degraded to glycine, which is also metabolized via the glycine decarboxylase-glycine reductase system if selenium is present (2, 13). *C. cylindrosporum* can only grow on glycine in the presence

of uric acid or some other purines, like *C. purinolyticum* (2, 12, 13), but requires higher concentrations of purines (H. Lebertz, Ph.D. thesis, University of Göttingen, 1984). The dihydrolipoamide dehydrogenase exhibited K_ms for NAD(P)H and NAD(P) which were about the same, contrary to those of the enzyme of *Eubacterium acidaminophilum*, which indicated a preference for NADP(H) (17). No isoenzyme could be detected during purification procedures, and both activities could not be separated. With the exception of reacting with both NADP(H) and NAD(H), the dihydrolipoamide dehydrogenase of *C. cylindrosporum* exhibited very similar structural properties to the enzyme of *Peptostreptococcus glycinophilus*. Contrary to the atypically small enzyme of *Eubacterium acidaminophilum*, which also reacts with both coenzymes (17), a transhydrogenase activity was catalyzed between NAD(P)H and thio-NAD(P)$^+$ in each of the combinations possible. Thus, electrons generated from glycine oxidation can be transferred to NADP$^+$ as well as to NAD$^+$. The enzyme methylenetetrahydrofolate dehydrogenase is NADP$^+$ specific (43). Formate dehydrogenase (as xanthine dehydrogenase, too) reacts best with methyl viologen, as also observed for *C. acidiurici* (45), which points to an involvement of ferredoxin (W. H. C. Bradshaw and D. J. Reeder, Bacteriol. Proc., p. 110, 1964). *C. acidiurici* contains an NADH:ferredoxin-oxidoreductase. NADPH could substitute for NADH if catalytic amounts of NADH were present (44). The authors concluded that a transhydrogenase reaction couples the NADH-specific and NADPH-specific processes. Perhaps the dihydrolipoamide dehydrogenase isolated is involved in transhydrogenation reactions.

The dihydrolipoamide dehydrogenase of *C. sporogenes* represented another new type (type Ic in Table 3). The enzyme did not use NAD(H); only NADP(H) was active, as found for both extracts and the homogeneous protein. The purified enzyme of *C. sporogenes* was a dimer of two identical subunits with a subunit M_r of about 52,000. Thus, the structural properties strongly resemble those of dihydrolipoamide dehydrogenases isolated from other organisms, but the coenzyme specificity is completely reversed in favor of NADP(H). To our knowledge, *C. sporogenes*, does not possess a pyruvate dehydrogenase, 2-oxoacid dehydrogenase, or glycine decarboxylase complex. In accordance with that, glycine is only reduced to acetate (8). There seems to be no need for a dihydrolipoamide dehydrogenase as found for halobacteria (9–11). After separating protein samples of *C. sporogenes* by SDS-polyacrylamide gel electrophoresis and transferring the proteins onto nitrocellulose filters by the Western blot technique, a double immunofluorescence labeling with anti-*Eubacterium acidaminophilum* dihydrolipoamide dehydrogenase IgG (17) gave rise to a fluorescent blot band with an M_r of 34,000, very close to the dihydrolipoamide dehydrogenase of *Eubacterium acidaminophilum* used as reference (M_r 34,500). During the process of purification, the cross-reacting protein was separated. The latter protein did not exhibit dihydrolipoamide dehydrogenase activity, although we tested it by various means. Thus, the cross-reaction observed in extracts of *C. sporogenes* (17) involved a protein other than dihydrolipoamide dehydrogenase.

The dihydrolipoamide dehydrogenases found in the other strictly anaerobic bacteria studied belonged to one of the types depicted in Table 3. The dihydrolipoamide dehydrogenases of *Eubacterium limosum* and *C. sticklandii* were similar to the enzyme of *Peptostreptococcus glycinophilus* (type Ia). The dihydrolipoamide dehydrogenase from the anaerobic cocci of Hare group IX strain NCTC 9811 was purified by us, too. It exhibited the same characteristics as the enzyme from *C. cylindrosporum*, acting on both coenzymes (type Ib). The organisms represented by type Ib have in common the ability to degrade purines and glycine. The dihydrolipoamide dehydrogenases of *Peptostreptococcus parvulus* and *Peptostreptococcus variabilis* seemed to belong to the group represented by *C. sporogenes* (type Ic). So far, our screening has failed to reveal other organisms which contained only the dihydrolipoamide dehydrogenase found in *Eubacterium acidaminophilum*, classified as type II.

The dihydrolipoamide dehydrogenase of *Eubacterium acidaminophilum* seems to be similar to a protein which is present in some other strictly anaerobic, amino acid-utilizing bacteria, but might have no primordial dihydrolipoamide dehydrogenase activity. The purification of such a protein from *C. sporogenes* was possible and resulted in a dimeric flavoprotein with a subunit M_r of about 35,000 exhibiting a high degree of conservation in the N-terminal amino acid sequence to the enzyme of *Eubacterium acidaminophilum* (D. Dietrichs and J. R. Andreesen, unpublished results). Further investigations have to be done to examine the involvement of some of these enzymes in the electron flow in reductase systems as found for *Eubacterium acidaminophilum* (18) and to compare their structural and functional relationships.

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