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Isolation of an Atypically Small Lipoamide Dehydrogenase Involved in the Glycine Decarboxylase Complex from *Eubacterium acidaminophilum*

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The lipoamide dehydrogenase of the glycine decarboxylase complex was purified to homogeneity (8 U/mg) from cells of the anaerobe *Eubacterium acidaminophilum* that were grown on glycine. In cell extracts four radioactive protein fractions labeled with D-[2-^{14}C]riboflavin could be detected after gel filtration, one of which coeluted with lipoamide dehydrogenase activity. The molecular mass of the native enzyme could be determined by several methods to be 68 kilodaltons, and an enzyme with a molecular mass of 34.5 kilodaltons was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot analysis of cell extracts separated by sodium dodecyl sulfate-polyacrylamide or linear polyacrylamide gel electrophoresis resulted in a single fluorescent band. NADPH instead of NADH was the preferred electron donor of this lipoamide dehydrogenase. This was also indicated by Michaelis constants of 0.085 mM for NADPH and 1.1 mM for NADH at constant lipoamide and enzyme concentrations. The enzyme exhibited no thioredoxin reductase, glutathione reductase, or mercuric reductase activity. Immunological cross-reactions were obtained with cell extracts of *Clostridium cylindrosporum*, *Clostridium sporogenes*, *Clostridium sticklandii*, and bacterium W6, but not with extracts of other glycine- or purine-utilizing anaerobic or aerobic bacteria, for which the lipoamide dehydrogenase has already been characterized.

Lipoamide dehydrogenase is known as an integral component of the pyruvate, 2-oxoglutarate, and branched-chain 2-oxoacid multienzyme complex (54). It catalyzes the reaction dihydrolipoamide + $NAD^+ \rightleftharpoons$ lipoamide + NADH + H^+. Glycine oxidation by bacteria, plants, and animals also involves a lipoamide dehydrogenase (15, 20, 21, 51). The glycine decarboxylase multienzyme complex consists of four proteins (traditionally called P1 to P4 in bacteria and P, H, L, and T, respectively, in animals) and catalyzes the reversible oxidation of glycine, yielding carbon dioxide, ammonium, 5,10-methylenetetrahydrofolate, and a reduced pyridine nucleotide. In this complex protein P1 binds glycine and releases the carboxyl group of glycine as CO_2 by transferring the remaining aminomethyl moiety to the lipoic acid group which is part of the small heat-stable protein P2. Protein P4 catalyzes the formation of 5,10-methylenetetrahydrofolate and ammonium from the aminomethyl group that is bound to P2. The electrons accepted by the lipoic acid group of protein P2 during glycine oxidation are generally transferred to NAD^+ by the lipoamide dehydrogenase protein P3 (for a review, see reference 20).

In *Escherichia coli* one gene codes for the lipoamide dehydrogenase protein which is part of both pyruvate and 2-oxoglutarate dehydrogenase (13). The nucleotide sequence predicts a somewhat smaller monomer (M_r, 51,274) (44) than that which is usually found by biochemical methods (M_r, 52,000 to 59,000) (13, 54). The DNA sequences for lipoamide dehydrogenase of yeast, porcine, and human origin contain, in addition, a leader sequence coding for 21 to 35 amino acids (5, 30, 33). In general, the enzyme contains 473 to 478 amino acids; thus, the calculated M_r without flavin adenine dinucleotide (FAD) varies between 49,436 and 51,556 regardless of its procaryotic or eucaryotic origin (4, 5, 14, 18, 30, 33, 50, 53, 55). The multiplicity of isoenzymes observed from eucaryotic origin was found to be a conformational isomerism (19). Immunological studies suggest that lipoamide dehydrogenase is identical in the pyruvate, 2-oxoglutarate, and branched-chain 2-oxoacid dehydrogenases of the rat heart (26).

In enzyme assays pig heart diaphorase is able to replace lipoamide dehydrogenase, protein L, from the chicken liver glycine decarboxylase (12). The lipoamide dehydrogenase of glycine decarboxylase from pea leaf mitochondria exhibits a monomer with an M_r of about 59,000, which is similar to that of the enzyme that is involved in the pyruvate dehydrogenase complex, and monoclonal antibodies raised against lipoamide dehydrogenase inhibit both enzymes to the same extent (51). Therefore, being a conservative enzyme, lipoamide dehydrogenase has also been used to calculate evolutionary relationships (9, 27) to other pyridine nucleotide disulfide oxidoreductase flavoproteins such as glutathione reductase, thioredoxin reductase, and mercuric reductase (11, 16, 36, 54).

As the only exception *Pseudomonas putida* and *Pseudomonas aeruginosa* produce two lipoamide dehydrogenases during growth on valine, which differ to some degree in molecular masses and kinetic parameters. One is part of 2-oxoglutarate dehydrogenase and probably pyruvate dehydrogenase; the other is part of branched-chain 2-oxoacid dehydrogenase (27, 39). During glycine oxidation by *P. putida*, only the lipoamide dehydrogenase of 2-oxoglutarate dehydrogenase is involved, which has a monomer molecular mass of 56 kilodaltons (kDa), however, rather than the enzyme specific for valine, which has a monomer molecular mass of 49 kDa (38).

From all these data it seemed that no specific lipoamide dehydrogenase is involved in glycine oxidation. However, during the isolation of the glycine decarboxylase proteins

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from *Eubacterium acidaminophilum*, an anaerobic bacterium that rapidly utilizes glycine (56), an unexpectedly low molecular mass of about 35 kDa for the monomer of lipoamide dehydrogenase protein P3 was detected in preliminary studies (H. Lebertz, Ph.D. thesis, University of Göttingen, Federal Republic of Germany, 1984). Thus, the objective of this study was to look for possible isoenzymes of lipoamide dehydrogenase by using radioactive riboflavin as an additional marker, to isolate the protein, and to compare some of its properties with those of other lipoamide dehydrogenases.

MATERIALS AND METHODS

Chemicals. D-[2-^{14}C]riboflavin was obtained from Amersham-Buchler (Braunschweig, Federal Republic of Germany). Enzymes and coenzymes were from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany). Fluorescein isothiocyanate goat-antirabbit-immunoglobulin G (IgG) was obtained from AHS Deutschland GmbH (Munich, Federal Republic of Germany); the molecular weight marker protein was from Sigma (Deisenhofen, Federal Republic of Germany). Sephadex G-100, DEAE-Sephacel, Sepharose CL-6B, CNBr-activated Sepharose 4B, and protein A-Sepharose CL-6B were purchased from Pharmacia (Freiburg, Federal Republic of Germany); DEAE-cellulose DE-52 was from Whatman Ltd. (Maidstone, United Kingdom); and hydroxylapatite Bio-Gel HTP was from Bio-Rad Laboratories (Munich, Federal Republic of Germany). DL-Dihydrolipoamide was prepared by the method of Reed et al. (31). All other chemicals were of the highest purity available and were obtained from commercial sources.

Organisms and growth conditions. *Eubacterium acidaminophilum* a1-2 (DSM 3953) was grown anaerobically on 25 mM glycine as described previously (56). The cells were mass-cultured in 20-liter carboys at 30°C and harvested by centrifugation (8,000 × *g*) while they were in the logarithmic growth phase. *Clostridium acidiurici* DSM 604, *Clostridium cylindrosporum* DSM 605, *Clostridium purinolyticum* DSM 1384, and *Eubacterium angustum* DSM 1984 were grown on the medium described by Dürre and Andreesen (10) by using uric acid (12 mM) plus glycine (100 mM) as the substrate. *Acetobacteroides glycinophilus* DSM 3078 and *Acidaminobacter hydrogenoformans* DSM 2748 were grown on glycine, as described previously (56), that was supplemented with 0.2% (wt/vol) yeast extract. Growth of other species was as described previously, as follows: *Clostridium sticklandii* DSM 519 (42), *Clostridium sporogenes* DSM 795 (29), *Pseudomonas putida* DSM 50202 (17), and *Arthrobacter globiformis* DSM 20124 (22). Media for *Azotobacter vinelandii* DSM 2289, *Escherichia coli* K-12 DSM 498, *Sporomusa ovata* DSM 2662, *Peptostreptococcus barnesae* DSM 3244, *Peptostreptococcus glycinophilus* DSM 20474, *Peptostreptococcus parvulus* DSM 20469, *Peptostreptococcus prevotii* DSM 20548, *Peptostreptococcus variabilis* ATCC 14955, Hare group III (NCTC 9803), and Hare group IV (NCTC 9804) are described in the Deutsche Sammlung von Mikroorganismen (DSM [German Collection of Microorganisms]), Catalogue of Strains (1986).

Preparations of cell extracts. A suspension of 10 g (wet weight) of cells was incubated in 20 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM dithioerythritol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg of DNase I, and 10 mg of lysozyme at 37°C for 15 min. The suspension was passed through a precooled French pressure cell at 147 MPa. The suspension of broken cells was centrifuged at 45,000 × *g* for 30 min at 4°C. The resulting supernatant (crude extract) was used.

Enzyme assays. The combined action of all four proteins of the glycine decarboxylase complex was measured in a final volume of 1 ml containing 60 mM potassium phosphate buffer (pH 7.8), 5 mM dithioerythritol, 2.5 mM tetrahydrofolate, 0.2 mM NAD(P)$^+$, 0.25 mM pyridoxal phosphate, and 50 mM glycine (21). Assays contained either crude extract or preparations of glycine decarboxylase proteins. The reaction was initiated by the addition of glycine, and the increase in A_{365} was followed with time. Specific activities (in units per milligram) are expressed as micromoles of NAD(P)H produced per minute and milligram of protein at 35°C. The activities of the lipoamide dehydrogenase protein P3 were measured (i) by the oxidation of NAD(P)H, in which the assay mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM dithioerythritol, 0.2 mM NAD(P)H, 1.5 mM lipoamide, and various amounts of enzyme preparations (49); (ii) by the reduction of NAD(P)$^+$ (2 mM) with 1.5 mM dihydrolipoamide in the same assay mixture; and (iii) by the transfer of electrons from NAD(P)H to benzylviologen with a concomitant increase in the A_{555} with time (21). The assay conditions were the same as those for method (i) given above, with the exception that lipoamide was replaced by 10 mM benzylviologen. Transhydrogenase and diaphorase activities of protein P3 were measured as described previously (49). Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities were determined as described previously (40). Thioredoxin reductase activity was determined as described by Clement-Metral (7); glutathione reductase activity was determined as described by Scrutton et al. (36).

Purification of protein P3. D-[2-^{14}C]riboflavin (0.6 μmol, 74 kBq) was added 2 h after inoculation of 800 ml of growth medium. The labeled cells were mixed with nonradioactive cells before the preparation of the cell extract. The purification procedure started with 1,050 mg of protein in the cell extract containing 44.4 kBq of radioactivity. The extract was applied to a column (2.6 by 30 cm) of DEAE cellulose (Whatman DE-52) that was previously equilibrated with 50 mM potassium phosphate buffer (pH 7.8) plus 1 mM dithioerythritol and 1 mM K_2Mg-EDTA (P3 buffer). The column was washed with 100 ml of this buffer and was then washed with a gradient of 0 to 1.0 M KCl in 200 ml of the same buffer at a flow rate of 30 ml/h. Lipoamide dehydrogenase was eluted at approximately 0.25 M KCl. The pooled enzyme solution was concentrated by ultrafiltration (Ultrafilter SM 145 49; Sartorius, Göttingen, Federal Republic of Germany) to a final volume of 7 ml. The concentrated enzyme solution was applied to a column (2.6 by 70 cm) of Sephadex G-100 that was equilibrated with P3 buffer, and the proteins were separated by using the same buffer at a flow rate of 10 ml/h. The pooled enzyme fractions were directly applied to a column (2.6 by 15 cm) of DEAE-Sephacel that was equilibrated with P3 buffer. The column was washed with 50 ml of this buffer and then with 50 ml of P3 buffer containing 0.1 M KCl. The enzyme was eluted with a gradient of 0.1 to 0.5 M KCl in 200 ml of P3 buffer at a flow rate of 30 ml/h. Fractions with lipoamide dehydrogenase activity were pooled and dialyzed at 4°C against 10 mM potassium phosphate buffer (pH 7.0) plus 1 mM dithioerythritol and 1 mM K_2Mg-EDTA. The dialyzed enzyme solution was applied to a column (1.5 by 6 cm) of hydroxylapatite that was equilibrated with the dialysis buffer. The column was washed with this buffer (20 ml) and then with a stepwise gradient of 50 ml each of 50, 70, and 100 mM P3 buffer at pH 7.0.

Electrophoresis. Fractions containing enzyme activity were tested for purity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (24) by using 10% polyacrylamide gels and a molecular weight marker kit (MW-SDS-70L; Sigma) in a 0.1 by 10 by 10 cm Minigel apparatus (Biometra, Göttingen, Federal Republic of Germany). Linear polyacrylamide gradient gel electrophoresis was performed as described previously (25) with a 4 to 27% polyacrylamide gradient. Electrophoresis was carried out at 100 V for 12 h with 5 mM Tris–38 mM glycine buffer. Lipoamide dehydrogenase activity was demonstrated in polyacrylamide gradient gels by using dihydrolipoamide (1.5 mM) as the electron donor and benzylviologen (8 mM) as the electron acceptor. Protein molecular weight estimation by native polyacrylamide gel electrophoresis under nondenaturing conditions was performed as described in Technical Bulletin MKR-137 (Sigma Chemical Co., St. Louis, Mo.).

Molecular weight determination. Analytical sucrose density gradient centrifugation was done with an ultracentrifuge (Spinco L2-65B; Beckman Instruments, Inc., Fullerton, Calif.) for 26 h at 75,000 × *g*. A linear sucrose gradient (2) of 3 to 27% sucrose was used. The molecular mass of the protein P3 was calculated by using bovine liver catalase (M_r, 240,000), rabbit muscle aldolase (M_r, 158,000), horseradish peroxidase (M_r, 40,000), and hen egg lysozyme (M_r, 14,400) as standards. The molecular mass of pure protein P3 was further determined by gel filtration chromatography on a Sephadex G-100 column (2.6 by 70 cm) by using hemoglobin from horse (M_r, 64,500), peroxidase, lysozyme, and blue dextran (BD 2000; Pharmacia, Uppsala, Sweden).

Thiol titration. The number of 5,5'-dithio-bis(2-nitrobenzoic acid)-titratable thiols in oxidized and NADPH-reduced protein P3 (4.5 mM) was determined under denaturing conditions as described previously (37).

Flavin identification. For identification of the flavin of protein P3, the enzyme was boiled for 15 min in the dark and centrifuged (15,000 × *g*, 15 min). The resulting supernatant was analyzed by thin-layer chromatography on thin-layer chromatographic cellulose plates (20 by 20 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) (34).

Determination of protein, protein spectra, and radioactivity. Protein was determined by the method of Bradford (3); protein spectra were obtained at room temperature with a double-beam spectrophotometer (Uvikon 810; Kontron Instruments, Eching, Federal Republic of Germany). Samples were measured against a reference containing the sample buffer.

Radioactivity was determined in a liquid scintillation counter (model LS 75,000; Beckman) by using Quickszint 212 (Zinsser, Frankfurt, Federal Republic of Germany) as scintillation fluid and was expressed as disintegrations per minute calculated from counts per minute by automatic quench compensation.

Immunological techniques. Antiserum was raised by subcutaneous injection of 300 μg of purified protein P3 together with Freund adjuvants into a rabbit (weight, 2.5 kg; age, 6 months). A booster injection with the same amount of antigen was given 2 weeks later. Ten days later the rabbit was bled. Preimmune serum was taken before immunization. Purification of IgG specific against protein P3 and double immunodiffusion tests were done as described previously (32). Crude extracts (10 to 50 μg of protein of each of the microorganisms indicated) served as samples for the analyses of cross-reactivity. Quantitation of protein P3 in crude extract samples (52) was done by using 1% (wt/vol) agarose in 0.09 M Tris–0.7 M glycine buffer (pH 8.6) and 40 μg of anti-P3 IgG per ml of agarose solution (56°C). Electrophoresis was carried out at 100 V for 4 h.

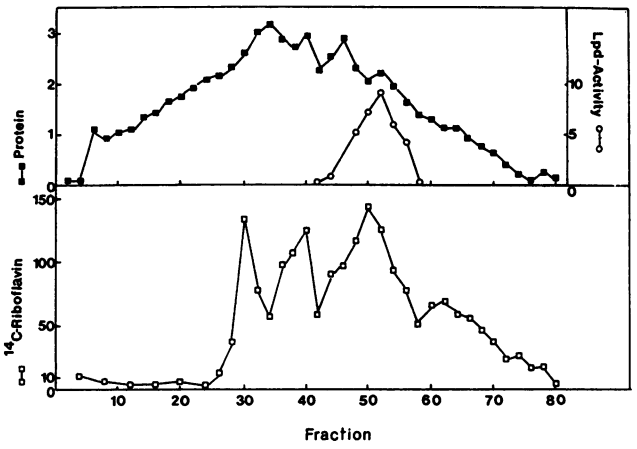

FIG. 1. Elution profile of D-[2-^{14}C]riboflavin labeled-proteins of *Eubacterium acidaminophilum*. Cells were grown in the presence of D-[2-^{14}C]riboflavin (0.75 μM, 74 kBq). A total of 5 ml of crude extract (220 mg) was applied to a Sepharose CL-6B column (2.6 by 100 cm) and fractionated at a flow rate of 10 ml/h. Fractions of 5 ml were collected. Protein activity is expressed as the A_{280}, and lipoamide dehydrogenase (lpd) activity is expressed as units per fraction. Radioactivity was determined as described in the text and is expressed as disintegrations per minute per 100 μl.

The specificity of IgG raised against protein P3 was demonstrated by double immunofluorescence labeling of blotted proteins (48). Protein samples separated by SDS- or linear polyacrylamide gel electrophoresis were transferred onto a nitrocellulose filter by the Western blot (immunoblot) technique. Binding of specific anti-P3 antibodies to the blotted protein and marking of specific antibodies by reaction with fluorescein isothiocyanate-labeled goat-antirabbit-IgGs were performed as described previously (23).

Chromatography on anti-P3–IgG–Sepharose. Protein A-Sepharose-purified anti-P3 IgGs (15 mg) were covalently bound to CNBr-activated Sepharose 4B (1 g) as described previously (Affinity Chromatography, Principles and Methods, Pharmacia Fine Chemicals, Uppsala, Sweden, 1983). Crude extract (200 mg) of *Eubacterium acidaminophilum* was applied to the antibody column (5 ml) with a flow rate of 0.1 ml/min followed by a washing step (50 ml of 0.5 M potassium phosphate buffer [pH 7.8]) to remove unspecific bound proteins. Protein P3 was eluted from the IgG-Sepharose by electroelution with a Biotrap BT 1000 (Schleicher & Schuell, Dassel, Federal Republic of Germany) with 5 mM Tris–38 mM glycine buffer (pH 8.3) at 200 V for 12 h.

RESULTS

Purification of protein P3. Before the purification procedure of the lipoamide dehydrogenase protein P3 was started, a portion of the radioactively labeled crude extract (5 ml) was fractionated on a Sepharose CL-6B column (2.6 by 100 cm) to determine the distribution of [2-^{14}C]riboflavin within the fractionated proteins and to look for possible isoenzymes of lipoamide dehydrogenase. Four radioactively labeled protein fractions could be detected, and the lipoamide-dependent enzyme activity of protein P3 coincided with one radioactivity peak (Fig. 1). No other fraction contained lipoamide dehydrogenase activity. One other protein fraction with a much larger molecular size exhibited activity by

TABLE 1. Steps in the purification of protein P3

Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Specific radioactivity (dpm/mg)	Recovery (%)	Purification (fold)
Cell extract	1,050	182	0.17	2.1×10^3	100	1.0
DEAE-cellulose	375	115	0.31	1.4×10^3	63	1.8
Sephadex G-100	84	80	0.96	2.9×10^3	44	5.5
DEAE-Sephacel	10	37	3.60	16.2×10^3	20	20.5
Hydroxylapatite	3	27	8.71	39.9×10^3	15	50.3

[a] Activity was determined by the reduction of lipoamide with NADPH. One unit is defined as 1 μmol of NADPH oxidized per min.

the unspecific NADH benzylviologen transfer assay; however, it was not active when lipoamide was used as the electron acceptor. During all purification steps, lipoamide dehydrogenase activity coeluted with radioactivity from the columns, and specific radioactivity increased, indicating a flavin content like that in all other lipoamide dehydrogenases (54). Four chromatographic steps were needed for purification of the enzyme to homogeneity (Table 1 and Fig. 2). The main difficulty encountered during the purification procedure was the separation of protein P3 from the lipoic acid-containing protein P2 of the glycine cleavage system. The latter protein should have been removed from the protein P3 fraction by gel filtration chromatography because of its low molecular mass (about 15 kDa). However, some of the protein P2 always eluted close to the protein P3 fraction up to the last purification step.

Attempts to purify the protein by affinity chromatography with an Affi-Gel Blue column (39) were unsuccessful because of insufficient binding to the affinity gel. However, when the anti-P3–IgG–Sepharose column was used, the enzyme was purified in one step.

The protein obtained after electroelution showed some unexpected differences to the conventionally purified enzyme regarding substrate specificity and spectroscopy (Fig. 3). When NADPH and lipoamide were used, the specific activity was only 50%, and no activity could be detected with dihydrolipoamide as the electron donor. The NADPH benzylviologen activity was in the range of the conventionally purified enzyme, reaching 18 U/mg.

Determination of purity and molecular mass. Electrophoresis of purified protein P3 on SDS-polyacrylamide gels resulted in a single protein band (Fig. 2), confirming the homogeneity of the protein fraction. An M_r of 34,500 was

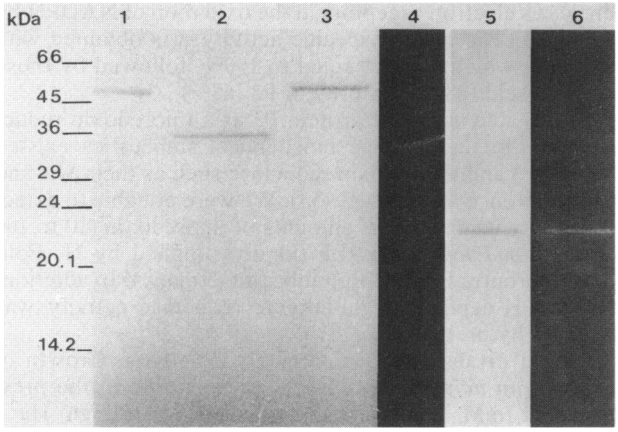

FIG. 2. Polyacrylamide gel electrophoresis of lipoamide dehydrogenases and blot analysis of crude extract of *Eubacterium acidaminophilum*. Conditions for electrophoresis and immunoblotting were described in the text. Lane 1 to 3, Proteins separated by SDS-polyacrylamide gel electrophoresis and determined by staining with Coomassie blue; lane 4, immunoblot; lanes 5 and 6, proteins separated by linear polyacrylamide gradient gel electrophoresis (4 to 27%); lane 5, lipoamide dehydrogenase as determined by activity stain; lane 6, immunoblot. Lane 1, 3 μg of lipoamide dehydrogenase isolated from *Clostridium cylindrosporum*; lane 2, 2.5 μg of protein P3; lane 3, 3 μg of lipoamide dehydrogenase isolated from *Peptostreptococcus glycinophilus*; lanes 4, 5, and 6, crude extract (15 μg) of *Eubacterium acidaminophilum*.

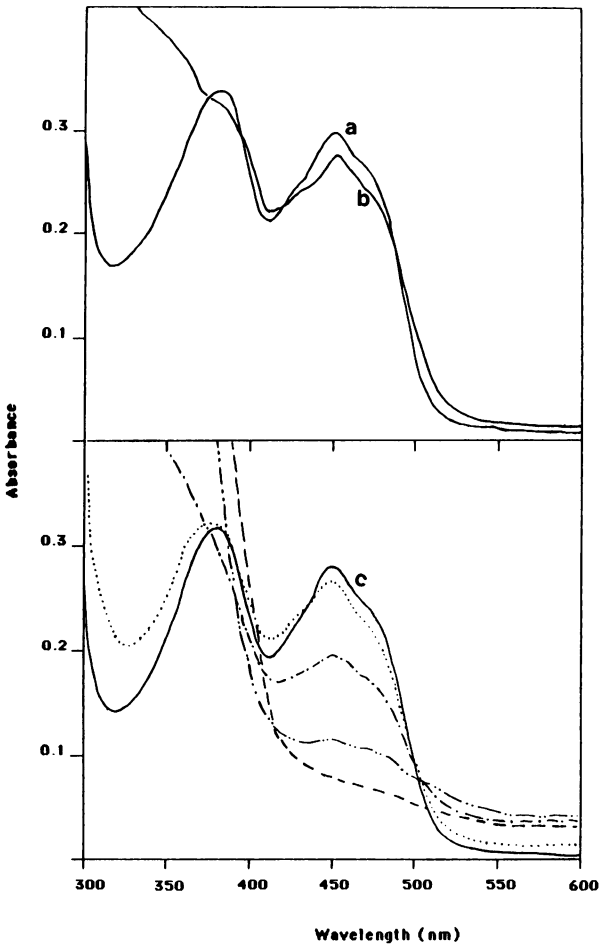

FIG. 3. Absorption spectra of protein P3. Spectra were taken anaerobically under an N_2 atmosphere. Curve a, Oxidized protein P3 (0.33 mg/ml) isolated by electroelution from an anti-P3–IgG–Sepharose column. Curve b, Conventionally isolated P3 (0.29 mg/ml). Curve c, Anaerobic titration of oxidized protein P3 (0.005 mM) with NADPH at 0.01 mM (· · · · · ·), 0.1 mM (– · – ·), 0.5 mM (· · – · ·), and 1.0 mM (– – – –).

calculated for the enzyme that was isolated by conventional and immunological techniques by comparing its electrophoretic mobility with those of standard proteins. This M_r was definitely smaller than the M_r obtained for lipoamide dehydrogenases isolated from *Clostridium cylindrosporum* and *Peptostreptococcus glycinophilus* (Fig. 2), which have M_rs of 50,000 and 52,000, respectively (D. Dietrichs, thesis of diploma, University of Göttingen, Federal Republic of Germany, 1987). Molecular mass estimations done by native polyacrylamide gel electrophoresis under nondenaturing conditions resulted in an M_r of about 70,000. To check this result, analytical sucrose density gradient centrifugation and gel filtration chromatography with Sephadex G-100 and standard proteins were also performed, resulting in M_rs of 65,000 and 68,000, respectively. Compared with the molecular mass of the SDS-denatured protein (34.5 kDa), the enzyme should consist of two identical subunits.

FAD content and spectra. The flavin of protein P3 was identified as FAD by thin-layer chromatography after heat treatment. An FAD content of 2.14 mol per native protein of 68 kDa was calculated from the supernatant after the enzyme was denatured by heat treatment and when an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm was used. From this result we also concluded that the molecular mass of protein P3 could not be higher assuming a flavin content of 1 mol of FAD per subunit. The P3 protein that eluted from the antibody column exhibited two defined maxima at 453 and 380 nm (Fig. 3, curve a), as has been typically observed for lipoamide dehydrogenases (54). An A_{280}/A_{450} ratio of 4.9 was calculated for this enzyme. However, the spectrum of oxidized protein P3 when it was purified by conventional techniques exhibited only one pronounced maximum at 453 nm, with shoulders at 445 and 477 nm (Fig. 3, curve b). Only a shoulder could be detected for the conventionally purified enzyme in the range of 380 nm. Anaerobic titration of the oxidized protein P3, which was purified either by the conventional or the immunological technique, with 1 mol of NADPH per mol of FAD resulted in a small decrease in the A_{453}. Further addition of NADPH up to 100 mol per mol of FAD yielded the completely reduced enzyme, as indicated by the absorbance at about 453 nm (Fig. 3, curve c).

Enzyme activities. Samples containing the glycine decarboxylase proteins P1, P2, and P4 showed no glycine decarboxylase activity. However, glycine decarboxylase activity could only be measured after the addition of protein P3, thus proving the involvement of the isolated protein P3 in glycine oxidation. In addition, no pyruvate or 2-oxoglutarate dehydrogenase activity was detectable in extracts of *Eubacterium acidaminophilum*; however, a pyruvate-ferredoxin-oxidoreductase was present (56). Glycine decarboxylase activity was the highest when $NADP^+$ instead of NAD^+ was used as the electron acceptor; NAD^+ resulted in only 34% of the glycine decarboxylase activity obtained with $NADP^+$. The preference of protein P3 for NADPH was further indicated by the difference in K_m values for NADPH and NADH of the lipoamide dehydrogenase protein P3 obtained at constant enzyme (0.7 μM) and lipoamide (1.5 mM) concentrations which were at least an order of magnitude lower for NADPH (0.085 mM) compared with that for NADH (1.1 mM). Pig heart lipoamide dehydrogenase was able to replace protein P3 in the glycine decarboxylase reaction, but it reacted only with NAD^+. The conventionally isolated enzyme had the highest activities when dihydrolipoamide was used as the electron donor and $NADP^+$ was used as the electron acceptor (Table 2). In contrast, the enzyme isolated by immunoabsorption was not able to catalyze this reaction and the NADPH-lipoamide reaction was decreased by 50%, but the NADPH-benzylviologen reaction was not. We suppose that the substrate binding site of the enzyme underwent changes during the interaction with the antibody and electroelution, for the enzyme still reacted with the unspecific substrate benzylviologen. The conventionally purified lipoamide dehydrogenase protein P3 catalyzed transhydrogenase reactions (Table 2). Again, NADPH was the preferred substrate. The enzyme was not able to catalyze a transhydrogenase reaction between, e.g., NADH and thio-$NADP^+$.

TABLE 2. Multifunctional enzyme activities of protein P3[a]

Reaction catalyzed by protein P3			Enzyme activity (U/mg)
NADH	⟶	lipoamide	1.5
NADPH	⟶	lipoamide	8.4
NADH	⟶	thio-NAD^+	0.6
NADH	⟶	thio-$NADP^+$	ND[b]
NADPH	⟶	thio-$NADP^+$	1.4
NADPH	⟶	thio-NAD^+	ND
NADH	⟶	benzylviologen	0.6
NADPH	⟶	benzylviologen	18.6
Dihydrolipoamide	⟶	NAD^+	1.1
Dihydrolipoamide	⟶	$NADP^+$	28.1

[a] Conventionally purified protein P3 was used. Activities were determined as described in the text.
[b] ND, Not detectable.

Reduction of lipoamide by NAD(P)H was stimulated when oxidized pyridine nucleotides were present in the assay system. Optimal enzyme activity was obtained at an $NADPH/NADP^+$ ratio of 100, resulting in an increase in the specific activity of about 37% compared with that of a control without added $NADP^+$. An $NADPH/NADP^+$ ratio of 5 decreased the enzyme activity by about 5%, and at a ratio of 1:1 only 56% of the activity remained.

Substrate specificity. Lipoamide (0.25 mM), α-lipoic acid (0.25 mM), and the natural substrate, the lipoic acid-containing protein P2 (50 μg), were compared for their efficiencies as electron acceptors in the oxidation of NADPH by protein P3. The highest specific activity was obtained with lipoamide, which was set equal to 100%, followed by those for lipoic acid (88%) and protein P2 (68%).

The possible action of protein P3 as a thioredoxin reductase was tested, for some biochemical similarities existed between P3 and thioredoxin reductase such as their M_rs and their preference for $NADP^+$ (54). We were not able to detect any activity using various amounts of thioredoxin (10 to 100 μg/ml) from *Escherichia coli* (kindly supplied by H. Follmann, Marburg, Federal Republic of Germany). In addition, no NADPH-dependent glutathione reductase activity was catalyzed by protein P3.

Effect of divalent cations on catalytic activity. Growth of *Eubacterium acidaminophilum* is still possible in the presence of 1 mM $HgCl_2$ (56). Because of the high Hg^{2+} tolerance and some biochemical similarities of protein P3 toward mercuric reductase (11), we wondered whether protein P3 was able to act as a mercuric reductase. The latter enzyme required an excess of exogenous thiols to prevent the formation of an inhibitory NADPH-Hg^{2+} complex and to ensure that Hg^{2+} is present as the dimercaptide. The protein P3 was dialyzed against buffer that was free of EDTA. No activity could be measured when lipoamide was replaced by $HgCl_2$ (0.1 mM) as the electron acceptor in the presence of 1 mM dithioerythritol. Surprisingly, no inhibition of lipoamide dehydrogenase activity was detected when lipoamide was added to this assay mixture. Therefore, we investigated the

influence of other divalent cations on lipoamide dehydrogenase activity. In the presence of dithioerythritol only Zn^{2+}, Ni^{2+}, and Fe^{2+} (0.1 mM each) slightly decreased the lipoamide dehydrogenase activity (by 22, 15, and 12%, respectively); but in the absence of dithioerythritol the enzyme activity was significantly inhibited by 0.1 mM Cu^{2+} (84%), Co^{2+} (59%), and Ni^{2+} (52%) and only slightly inhibited by Fe^{2+} (17%) and Ca^{2+} (20%). No inhibition was measured in the presence of 0.1 mM Zn^{2+} or Mg^{2+}. In the absence of dithioerythritol, the spontaneous reaction of Hg^{2+} with NADPH prevented a study of the influence of Hg^{2+} on protein P3 activity by this test system. To avoid this effect, protein P3 was incubated with 0.1 and 0.01 mM $HgCl_2$ in the absence of dithioerythritol at 30°C for 10 min; then 1 mM dithioerythritol was added to bind free Hg^{2+} ions. Lipoamide dehydrogenase was still active after this treatment, exhibiting activities of 49 and 78%, respectively.

Involvement of redox-active disulfide. A redox-active disulfide should be present in the enzyme, if the catalytic mechanism of protein P3 is similar to that of other lipoamide dehydrogenases (54). Both oxidized and NADPH-reduced enzymes were denatured with guanidine hydrochloride and reacted with 5,5'-dithio-bis(2-nitrobenzoic acid). The number of thiols calculated for the oxidized enzyme was 2.6, whereas with the NADPH-reduced enzyme 4.2 thiols were titratable. This difference of 1.6 thiols indicates a redox-active disulfide at the active site of protein P3.

Incubation of NADPH-reduced protein P3 with 1 mM phenylarsinoxide for 10 min in the absence of dithioerythritol and starting the test by adding lipoamide or benzylviologen led to the loss of 89 and 74% activity, respectively, compared with the activities of controls. Thus, phenylarsinoxide seems to react with the sulfhydryl groups of the enzyme that is involved in enzyme catalysis (45). These results emphasize the role of the sulfhydryl groups in the catalysis of the enzyme. The use of dithioerythritol in the standard assay indicated that reproducible enzyme activities can be achieved; these activities were about 60% higher than those in the absence of dithioerythritol.

Immunological studies. The specificity of the isolated antibodies against protein P3 was proved by the Western blot technique. After proteins from crude extract were separated by SDS-polyacrylamide gel electrophoresis, only one protein with a molecular mass of about 35 KDa reacted with anti-P3 IgGs (Fig. 2), confirming its specificity against protein P3 and the absence of immunologically related isoenzymes. Crude extracts of *Eubacterium acidaminophilum* separated by polyacrylamide gradient gel electrophoresis were subjected to a staining for lipoamide dehydrogenase activity, with dihydrolipoamide used as the electron donor and benzylviologen used as the electron acceptor under anaerobic conditions. The polyacrylamide gels showed only one blue-stained band, which also reacted with anti-P3 IgGs after the proteins were blotted onto nitrocellulose sheets (Fig. 2), which further proved the absence of related isoenzymes.

Anti-P3 antibodies were used to examine the possible immunochemical cross-reactions in crude extracts of various glycine- or purine-utilizing bacteria by double immunodiffusion tests. Cross-reactions could only be detected with extracts of *Clostridium cylindrosporum* (but not with the isolated lipoamide dehydrogenase of *Clostridium cylindrosporum* [D. Dietrichs, unpublished data]), *Clostridium sporogenes*, *Clostridium sticklandii*, or bacterium W6 (a betaine-utilizing anaerobe isolated by C. Fendrich [Göttingen, Federal Republic of Germany] from North Sea mud), but not with extracts of the mostly glycine-utilizing anaerobes (*Clostridium acidiurici*, *Clostridium purinolyticum*, *Sporomusa ovata*, *Peptostreptococcus glycinophilus*, *Peptostreptococcus parvulus*, *Peptostreptococcus prevotii*, *Peptostreptococcus variabilis*, Hare groups III and IV, *Eubacterium angustum*, *Eubacterium limosum*, *Acidaminobacter hydrogenoformans*, and *Acetobacteroides glycinophilus* or the aerobically grown *Escherichia coli*, *Pseudomonas putida*, *Arthrobacter globiformis*, or *Azotobacter vinelandii*, whose lipoamide dehydrogenases have been studied previously (4, 13, 22, 38). Antibodies raised against the lipoamide dehydrogenases of *Peptostreptococcus glycinophilus* and *Clostridium cylindrosporum* do not react with extracts of *Eubacterium acidaminophilum* or with the purified protein P3 (D. Dietrichs, unpublished data). The amount of protein P3 was quantified in crude extracts of *Eubacterium acidaminophilum* by rocket immunoelectrophoresis. By using a standard titration with purified protein P3 (0.08 to 1.2 μg), the content of protein P3 was calculated to be 1.4% of the soluble extract of *Eubacterium acidaminophilum*.

DISCUSSION

To our knowledge this is the first report of an atypically small lipoamide dehydrogenase which is involved in the glycine decarboxylase complex. According to expectations (1, 15, 21, 38, 51, 54), the lipoamide dehydrogenase from the glycine decarboxylase complex of *Eubacterium acidaminophilum* should be equivalent in size to those of the pyruvate and 2-oxoglutarate dehydrogenase complexes, as has been observed previously for *Pseudomonas putida* (38). As the exception, *P. putida* produces two lipoamide dehydrogenases during growth on valine (39), one (valine lipoamide dehydrogenase) is part of the branched-chain 2-oxoacid dehydrogenase; the other (glucose lipoamide dehydrogenase) is part of the pyruvate and 2-oxoglutarate dehydrogenase as well as the glycine decarboxylase (38). No specific lipoamide dehydrogenase seems to be present in the glycine cleavage system of *Escherichia coli* (43), for *Escherichia coli* posseses only a single structural gene for lipoamide dehydrogenase (44) and, thus, should have a single lipoamide dehydrogenase protein. From data presented previously (1, 21) and from results of studies in our laboratory (Dietrichs, thesis of diploma) (Fig. 2), it seems that protein P3 from the glycine-utilizing anaerobe *Peptostreptococcus glycinophilus* and the purinolytic *Clostridium cylindrosporum* are like the usual types of lipoamide dehydrogenases that are isolated, indicating that there is no general deviation of the enzyme that is specifically involved in anaerobic glycine metabolism. Anti-P3 antibodies did not show a cross-reaction with either crude extracts of *Eubacterium limosum*, the type species of the genus *Eubacterium*, or extracts of the glycine-utilizing *Eubacterium angustum*, indicating that the structure of protein P3 is unique among species belonging to the genus *Eubacterium*.

So far the most important differences observed between protein P3 of *Eubacterium acidaminophilum* and the usual type of lipoamide dehydrogenase were its low molecular mass and its preference for $NADP^+$, two formal aspects which protein P3 shares only with thioredoxin reductase, considering all the other known enzymes of the class of FAD-containing pyridine nucleotide disulfide oxidoreductases (11, 16, 28, 37, 46, 47, 54). However, no thioredoxin reductase activity could be detected for protein P3. Extracts of *Eubacterium acidaminophilum* contain only very low thioredoxin reductase activity (H. Follmann, personal communication).

The molecular masses of native lipoamide dehydrogenases from archaebacterial, eubacterial, and eucaryotic organisms are about 110,000 when determined by biochemical methods (4, 8, 13, 18, 35, 50, 53, 54). The only exception is the valine lipoamide dehydrogenase from *Pseudomonas putida* (M_r, 98,000) (40), which comes close to the molecular mass of 2 × 50,225 to 52,278 (100,450 to 104,556) determined from the nucleotide sequence of the enzymes of procaryotic and eucaryotic (without the leader peptide) origin (5, 30, 33, 44, 53). All these enzymes are dimers that contain 1 mol of FAD per subunit (54). Protein P3 of *Eubacterium acidaminophilum* also consisted of two identical subunits, and it seemed likely that it also contained 1 mol of FAD per subunit. However, a molecular mass of about 68 kDa was obtained for the native enzyme by various methods. This is too small to reflect a deviation of the regular size by the methods used, which usually cause it to be too large (44, 53). Like thioredoxin reductase (16, 28), two redox-active centers (the disulfide and FAD) and the two binding domains (for NADPH and the lipoamide-containing protein P2) must be accommodated on each enzyme subunit of 34.5 kDa, in contrast to 50 kDa for the well-known type examined thus far. The low specific activities of about 9 and 28 U/mg determined with the NADPH-lipoamide and dihydrolipoamide-NADP test systems, respectively, were another difference in the isolated lipoamide dehydrogenase of *Eubacterium acidaminophilum* compared with the usual lipoamide dehydrogenases (35). The activity of the lipoamide dehydrogenase of *Pseudomonas putida* (glucose lipoamide dehydrogenase) reaches 65 U/mg (39); that of *Peptostreptococcus glycinophilus* is at least 85 U/mg (1), whereas the enzyme from *Escherichia coli* exhibits activity of 140 U/mg (13). The enzyme from *Bacillus subtilis* exhibits a comparable low specific activity of 11 U/mg (35). However, the low activity of protein P3 from *Eubacterium acidaminophilum* might be compensated for by the relatively high amount of this protein within the cell (1.4%). The latter value correlated well with the purification factor (50.3-fold) obtained by conventional techniques. Therefore, the percentage of inactivated enzyme should be low.

An astonishing fact was the lowered or abolished lipoamide- or dihydrolipaomide-dependent enzyme activity, respectively, of the enzyme that was purified by immunoabsorption, although this enzyme preparation exhibited a spectrum that is typical for lipoamide dehydrogenases (54). Another peculiarity of the isolated enzyme was the resistance of the flavin moiety toward its reduction by reduced pyridine nucleotides. This can usually be accomplished with equimolar concentrations (54).

So far no lipoamide dehydrogenase has been described which preferentially reacts with $NADP^+$. During anaerobic glycine metabolism, 1 mol of glycine is completely oxidized to CO_2 by glycine decarboxylase, and the following oxidizing enzymes, such as methylenetetrahydrofolate dehydrogenase and formate dehydrogenase, are $NADP^+$ specific or at least prefer $NADP^+$ (56). The reducing equivalents that are generated are reoxidized by the action of glycine reductase, which exerts a higher activity when NADPH is used as the electron donor than when NADH is used as the electron donor (56; M. Rieth, Ph.D. thesis, University of Göttingen, Federal Republic of Germany, 1987). Therefore, it seems reasonable that glycine decarboxylase protein P3 preferentially reacted with $NADP^+$, thus allowing a directed electron flow toward glycine reduction. Perhaps some of the atypical properties of the lipoamide dehydrogenase protein P3 might be a reflection of its function to channel reducing equivalents from the glycine decarboxylase to the glycine reductase complex. In *Eubacterium acidaminophilum* glycine serves as both oxidant and reductant. An immunological cross-reaction observed between anti-P3 IgG and crude extracts of *Clostridium cylindrosporum*, *Clostridium sticklandii*, and *Clostridium sporogenes* indicated the presence of a protein which seems to be closely related to protein P3. However, in *Clostridium sticklandii* and *Clostridium sporogenes*, glycine was only utilized as an electron acceptor, thus allowing only a function of the related protein in the reductase system. Two flavoproteins were partially purified from *Clostridium sticklandii* which functioned as electron transport proteins in Stickland reactions, one of which was $NADP^+$ specific (41). Thus, protein P3 might function as a direct link between glycine oxidation and reduction, which may explain its different properties compared with those of other known lipoamide dehydrogenases. Recently, some evidence was obtained that rat liver mitochondria contain two immunologically distinct lipoamide dehydrogenases, one of which might be specifically involved in the glycine cleavage system (6).

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