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# Purification and Partial Characterization of the Glycine Decarboxylase Multienzyme Complex from *Eubacterium acidaminophilum*

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**The proteins P1, P2, and P4 of the glycine cleavage system have been purified from the anaerobic, glycine-utilizing bacterium *Eubacterium acidaminophilum*. By gel filtration, these proteins were determined to have $M_r$s of 225,000, 15,500, and 49,000, respectively. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein P1 was determined to have two subunits with $M_r$s of 59,500 and 54,100, indicating an $\alpha_2\beta_2$ tetramer, whereas the proteins P2 and P4 showed only single bands with estimated $M_r$s of 15,500 and 42,000, respectively. In reconsitution assays, proteins P1, P2, P4 and the previously reported lipoamide dehydrogenase (P3) had to be present to achieve glycine decarboxylase or synthase activity. All four glycine decarboxylase proteins exhibited highest activities when $NADP^+$ was used as the electron acceptor or when NADPH was used as the electron donor in the glycine synthase reaction. The oxidation of glycine depended on the presence of tetrahydrofolate, dithioerythreitol, $NAD(P)^+$, and pyridoxal phosphate. The latter was loosely bound to the purified protein P1, which was able to catalyze the glycine-bicarbonate exchange reaction only in combination with protein P2. Protein P2 could not be replaced by lipoic acid or lipoamide, although lipoic acid was determined to be a constituent (0.66 mol/mol of protein) of protein P2. Glycine synthase activity of the four isolated proteins and in crude extracts was low and reached only 12% of glycine decarboxylase activity. Antibodies raised against P1 and P2 showed cross-reactivity with crude extracts of *Clostridium cylindrosporum*.**

The glycine decarboxylase multienzyme complex has been investigated in various animals, plants, and bacteria (9, 12, 16, 20, 28, 33, 35) and catalyzes the reversible cleavage of glycine to form one molecule each of methylene-tetrahydrofolate (methylene-$H_4$-folate), carbon dioxide, and ammonia. Investigations performed so far have determined that this multienzyme complex always consists of four proteins, which traditionally have been named P, H, L, and T proteins when isolated from eucaryotic sources (16) or proteins P1 to P4, respectively, when isolated from bacteria (9, 17, 21). Glycine decarboxylase complex isolated from humans also consists of four proteins, and deficiency of one of these proteins leads to nonketotic hyperglycinaemia (15, 32).

Protein P1 usually is a pyridoxal phosphate-containing protein which binds glycine via a Schiff base and catalyzes the release of $CO_2$ from glycine. The remaining aminomethyl moiety is bound to the lipoic acid group of protein P2 and serves as a substrate for protein P4, which catalyzes the release of ammonia from the aminomethyl group and transfers the remaining one-carbon unit to $H_4$-folate to form 5, 10-methylene-$H_4$-folate. In the absence of $H_4$-folate, the protein P4 forms ammonia and formaldehyde as products from the aminomethyl group at a slower reaction rate (8). During the above-mentioned reactions, protein P2 is reduced and is subsequently reoxidized by protein P3, a flavin-containing lipoamide dehydrogenase which transfers the electrons finally to $NAD(P)^+$. The proteins P1 and P2 jointly catalyze the glycine bicarbonate exchange reaction, whereby exogenous $CO_2$ can replace the glycine carboxyl group. The P protein (P1), as isolated from animal liver, is able to catalyze this exchange reaction to 1% in the absence of H protein (P2) compared with in the presence of H protein (12).

The proteins of glycine decarboxylase complexes from various sources seem to be quite similar in structure and catalytic properties, as indicated by (i) the observation of immunological cross-reactivity among P proteins from various animal species (16) and (ii) the fact that in the glycine-bicarbonate exchange reaction, P and H proteins, for example from chicken liver, can each be replaced by the corresponding protein even from *Arthrobacter globiformis* (7, 10). So far, only the glycine decarboxylase/synthase of the purine-degrading anaerobic specialist *Clostridium acidiurici* seems to exhibit some dissimilarities in structure and catalytic properties, especially of protein P1. This has been explained by the different physiological role of this enzyme, which can function to a high degree both in the direction of glycine synthesis and in the direction of its degradation (9).

The anaerobic bacterium *Eubacterium acidaminophilum* utilizes glycine only in the presence of selenium in the growth medium. The key enzymes for its utilization are glycine decarboxylase and glycine reductase (37). The oxidation of 1 mol of glycine to 2 mol of $CO_2$ and 1 mol of $NH_3$ by glycine decarboxylase and other enzymes delivers the reduction equivalents for the further reduction of 3 mol of glycine to acetate via glycine reductase (4). In contrast, during growth on serine the glycine decarboxylase has to act as glycine synthase, forming glycine from $CO_2$, for no significant serine hydroxymethyltransferase activity can be measured (37). It is conceivable that this system in concert with the glycine reductase could enable some anaerobic bacteia to grow autotrophically if a hydrogenase were present (3, 6, 36). Although conditions for growth on $H_2$ and $CO_2$ have not yet been found, *E. acidaminophilum* possesses a hydrogenase and exhibits high and stable glycine reductase activity in crude extracts (37), which both constitute (in

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combination with the glycine synthase reaction) the key enzymes for acetate formation via this glycine pathway. In addition, the organism posseses an atypically small lipoamide dehydrogenase involved in the glycine decarboxylase complex (5). Therefore, it was the purpose of this study to isolate all the other proteins of glycine decarboxylase of this organism to show their interaction with the atypically small lipoamide dehydrogenase and to compare their basic properties with those of other organisms.

## MATERIALS AND METHODS

**Chemicals.** All radioactive chemicals were obtained from Amersham-Buchler, Braunschweig, Federal Republic of Germany. Enzymes and coenzymes were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. Molecular weight marker proteins were obtained from Sigma, Deisenhofen, Federal Republic of Germany; Sephadex G-100, DEAE-Sephacel, and Sepharose CL-6B were obtained from Pharmacia, Freiburg, Federal Republic of Germany; DEAE-cellulose DE-52 was obtained from Whatman Ltd., Maidstone, United Kingdom. All other chemicals were of the highest purity available from commercial sources.

**Organisms.** *E. acidaminophilum* al-2 (DSM 3952, ATCC 49065) was cultivated on glycine (37). *Escherichia coli* W 1485 lip2 (11), a gift from J. R. Guest, Sheffield, United Kingdom, and the bacteria used for immunological tests were cultivated as described previously (5).

**Enzyme assays.** The glycine decarboxylase enzyme complex was assayed either by the method of Motokawa and Kikuchi (27) by measuring the $^{14}CO_2$ as a reaction product from [1-$^{14}$C]glycine or by the method of Klein and Sagers (19) in an optical test. Mixtures contained (in a final volume of 1 ml): 68 mM potassium phosphate buffer, pH 7.8, 5 mM dithioerythreitol (DTE), 2 mM $H_4$-folate, 2 mM NAD(P), 0.25 mM pyridoxal phosphate, and various amounts of protein. Assays were performed anaerobically under $N_2$ atmosphere in cuvettes sealed with serum stoppers and were started with 50 mM glycine. Increase in $A_{365}$ was measured at 30°C.

Protein P1 activity was determined by glycine-$CO_2$ exchange assay (27). The assay mixtures were supplemented with crude preparations of P2 which had previously been incubated for 10 min at 80°C.

Activities of proteins P2 and P3 were measured as described previously (5, 27). Protein P4 had to be tested by a complementation test using the assay for the enzyme complex by adding pig heart diaphorase (0.4 U) instead of P3 (19).

Glycine synthase activity was determined anaerobically in vials sealed with serum stoppers containing (in a final volume of 1 ml): 50 mM potassium phosphate buffer, pH 7.8, 0.25 mM pyridoxal phosphate, 10 mM DTE, 5 mM formaldehyde, 2.5 mM $H_4$-folate, 5 mM $NH_4Cl$, 0.5 mM NAD(P)H, and 10 mM $NaH^{14}CO_3$ (0.5 μCi). The mixture was incubated for 1 h at 0°C to form methylene-$H_4$-folate and was warmed up to 30°C, and the reaction was started by adding crude extract or all four glycine decarboxylase proteins. The reaction was stopped by adding 100 μl of a 20% trichloroacetic acid solution. Glycine content was determined by liquid scintillation after incubation of the samples at 80°C for 10 min and identified by chromatography of the dansylated amino acid on DC-Micropolyamide sheets (Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany) (23). Units of enzyme activities were defined as micromoles of substrate used per minute at 30°C.

**Purification of glycine decarboxylase proteins.** Preparation of cell extracts was done as described previously (37).

**(i) Purification of protein P1.** Cell extract (32 ml [1,260 mg of protein]) was fractionated with solid ammonium sulfate. The 40% saturation precipitate was discarded, and the 70% saturation precipitate was dialyzed against 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTE and 0.25 mM pyridoxal phosphate (P1-buffer). The dialyzed protein fraction was applied to a Whatman DE-52 column (1.5 by 5 cm) equilibrated with P1-buffer. P1 eluted at approximately 180 mM KCl with a 200-ml linear gradient of 0 to 1 M KCl in P1-buffer. P1-containing fractions were pooled and ultrafiltrated to a volume of 8 ml, which was applied to a Sepharose CL-6B column (2.6 by 90 cm) equilibrated with P1-buffer. The pooled P1-containing fractions were applied to a second Whatman DE-52 column as described above and eluted with a linear gradient of 0 to 300 mM KCl in 200 ml of P1-buffer. The pooled and ultrafiltrated P1 fraction was subjected to a sucrose density centrifugation with a sucrose gradient from 6 to 28% sucrose in P1-buffer and centrifuged at 74,000 × *g* for 28 h. After centrifugation, each centrifuge tube (36 ml) was fractionated from the bottom, and 0.5-ml fractions were taken. Fractions containing P1 activity were applied to a DEAE-Sephacel column (1.5 by 5 cm) equilibrated with P1-buffer. P1 eluted at 220 mM KCl in a 200-ml gradient of 0 to 300 mM KCl.

**(ii) Purification of protein P2.** Cell extract was fractionated with solid ammonium sulfate. The 40 to 70% ammonium sulfate fraction was used for further purification of proteins P1 and P4; the 70 to 100% ammonium sulfate fraction was dissolved in 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM $K_2$Mg-EDTA (P2-buffer) and dialyzed against the same buffer. The dialyzed protein fraction was applied to a Whatman DE-52 column (1.5 by 5 cm) equilibrated with P2-buffer. P2 activity eluted at 550 mM KCl with a linear gradient from 0 to 1 M KCl in 200 ml of P2-buffer. Protein P2-containing fractions were dialyzed against P2-buffer, and a second ion-exchange chromatography was done on DEAE-Sephacel equilibrated with P2-buffer. The column was washed with 300 mM KCl in 50 ml of P2-buffer, and P2 was eluted with a linear gradient from 300 to 700 mM KCl in 200 ml of P2-buffer. The most active fractions were pooled, ammonium sulfate (5% wt/vol) was added, and the fraction was kept at 90°C for 10 min. The solution was centrifuged at 40,000 × *g* to remove precipitations. Purification of protein P3 was done as described previously (5).

**(iii) Purification of protein P4.** The 30 to 50% ammonium sulfate fraction was dialyzed against P1-buffer and applied to a Whatman DE-52 column as described above. By washing the column with 40 ml of P1-buffer, 73% of protein P4 activity was eluted. This protein P4 fractions was used for further purification by bringing it to 25% ammonium sulfate saturation and applying the solution to a Phenyl-Sepharose column (0.5 by 6 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTE and 25% ammonium sulfate. After washing the column with 50 ml of equilibration buffer, protein P4 was eluted with a decreasing linear gradient of 25 to 0% ammonium sulfate and decreasing ion force from 100 to 25 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTE. Protein P4 eluted in a broad peak between 14 and 6% ammonium sulfate saturation. Protein P4 fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.0) plus 1 mM DTE and applied to a column of Sepharose 4B containing covalently bound folate (0.5 by 5 cm) (29). About 80% of applied protein was bound to the column, but not protein P4, which was eluted by

washing the column with equilibration buffer. This fraction was applied to a hydroxylapatite column (0.5 by 2 cm) equilibrated with equilibration buffer. Proteins were eluted by a stepwise gradient of 10, 20, 50, 100, and 250 mM of that buffer. The main P4 activity eluted at 100 mM phosphate buffer and was dialyzed against 50 mM potassium phosphate buffer (pH 7.8) plus 1 mM DTE.

**Determination of lipoic acid content.** Lipoic acid was determined by the growth response of an *E. coli* mutant after lipoic acid was released from protein P2 by autoclaving the protein in 6 N $H_2SO_4$ (11). For calculation of the α-*dl*-lipoic acid content, a molecular mass of 15,500 for protein P2 was used. A standard curve was taken in the range of 1 to 10 ng of *dl*-lipoic acid.

**Immunological methods.** Preparing and isolation of specific immunoglobulins G (IgGs), Ouchterlony double immunodiffusion tests, and rocket immunoelectrophoresis were done as described previously (5).

**Other methods.** Molecular weight estimation were done on a Sephadex G-100 column (1.5 by 70 cm) for proteins P2 and P4, on a Sepharose CL-6B column (2.6 by 90 cm) for protein P1, and by analytical sucrose density centrifugation with a linear gradient from 5 to 28% sucrose (5).

Protein was determined by the method of Bradford (2) using bovine serum albumin as the standard.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was usually performed by the method of Laemmli (24), using 7.5, 10, and 14% polyacrylamide gels.

*E. acidaminophilum* cells were permeabilized in anaerobe P1-buffer by the method of Miozarri et al. (26). Specific activities of enzymes in permeabilized cells were calculated on the basis of an assumed 100% permeabilization by using a dry-weight-to-protein-content ratio of 2.

Radioactivity and absorption spectra of isolated proteins were determined as described before (5).

## RESULTS

**Isolation of the glycine decarboxylase proteins.** Cell extracts of *E. acidaminophilum* exhibited a stable glycine decarboxylase activity, but when crude extract was fractionated on gel filtration or by sucrose density centrifugation, no fraction exhibiting glycine decarboxylase activity could be detected. To characterize the enzyme complex, it was necessary to separate the component enzymes of the glycine cleavage system.

The proteins P1 and P2 could be separated by fractionated ammonium sulfate precipitation. The highest glycine-bicarbonate exchange reaction could be measured by combination of the fractions containing 40 to 60% and 75 to 100% saturated ammonium sulfate; no single fraction was able to catalyze this reaction. The protein P1 was purified 87-fold to homogeneity by six purification steps (Table 1). The enzyme activity was somewhat unstable in the purified form. Freezing and thawing of the purified protein in P1 buffer led to a decrease of about 60% in P1 activity. Purified protein P1 had to be stored in 10% glycerol, which, however, had to be removed from the protein P1 fraction by dialysis because glycerol caused a decrease in enzyme activity of about 45%, as did sucrose. During enrichment procedures it was necessary to supplement all buffers with pyridoxal phosphate in order to keep the enzyme active. Pyridoxal phosphate was removed from the purified enzyme by extensive dialysis against phosphate buffer plus 250 mM KCl as evidenced by the change in the spectrum (Fig. 1) accompanied by a total loss in enzyme activity. The latter could not be reactivated by addition of pyridoxal phosphate. Analysis of the purified protein P1 by SDS-polyacrylamide gel electrophoresis revealed two protein bands with $M_r$s of 59,500 and 54,100. The $M_r$ of 225,000 (± 8,000) for the native protein, as determined by gel filtration and sucrose density centrifugation, suggested the existence of protein P1 as an $\alpha_2\beta_2$ tetramer.

The protein P2 fraction obtained from the 75 to 100% saturated ammonium sulfate precipitate was purified 10-fold by its activity in the enzymatic assay (Table 1). The apparent

TABLE 1. Purification of proteins P1, P2, and P4 from *E. acidaminophilum*

| Protein and purification procedure | Total U | Protein (mg) | Activity (U/mg)[a] | Purification (fold) |
|---|---|---|---|---|
| P1 | | | | |
| Crude extract | 15.1 | 1,260 | 0.012 | 1 |
| Supernatant-40% AS[b] | 13.1 | 842 | 0.015 | 1.3 |
| DE-52 | 9.5 | 71 | 0.135 | 11.3 |
| Sepharose CL-6B | 7.0 | 22 | 0.320 | 26.6 |
| DE-52 | 5.1 | 8.3 | 0.621 | 51.7 |
| Sucrose density gradient centrifugation | 3.4 | 3.4 | 0.942 | 78.5 |
| DEAE-Sephacel | 1.6 | 1.5 | 1.051 | 87.5 |
| P2 | | | | |
| Crude extract | 96 | 1,476 | 0.065 | 1 |
| 70–100% AS[b] | 15 | 73 | 0.206 | 3.2 |
| DE-52 | 5.6 | 13.4 | 0.418 | 6.4 |
| DEAE-Sephacel | 4.4 | 7.1 | 0.620 | 9.5 |
| Heat treatment | 2.8 | 4.1 | 0.682 | 10.5 |
| P4 | | | | |
| 30–50% AS[b] | 30.7 | 640 | 0.048 | 1 |
| DE-52 | 20.2 | 192 | 0.105 | 2.2 |
| Phenyl-Sepharose | 13.2 | 62 | 0.213 | 4.4 |
| Folate-Sepharose 4B | 6.9 | 8.6 | 0.802 | 17 |
| Hydroxylapatite | 2.8 | 2.9 | 0.966 | 20 |

[a] Activities were determined as described in Materials and Methods.
[b] AS, Ammonium sulfate.

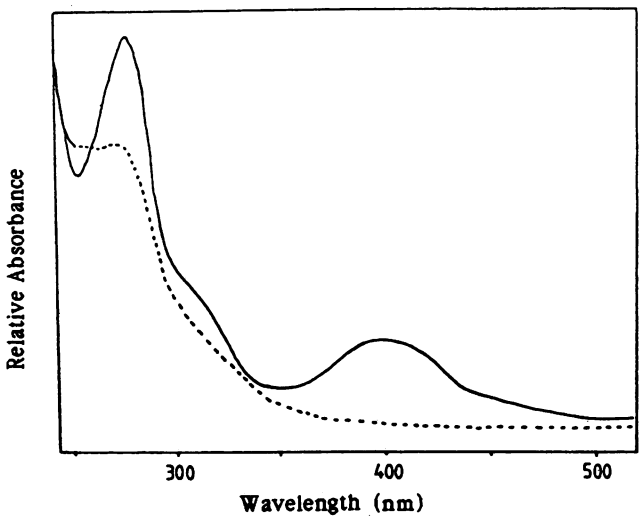


FIG. 1. Absorption spectra of protein P1. The spectra of isolated protein P1 were determined (i) in P1 buffer against a reference containing the same buffer (———) and (ii) after extensive dialysis of protein P1 against 50 mM potassium phosphate buffer containing 250 mM KCl (----). The reference contained the dialysis buffer.

TABLE 2. Requirement of cofactors in glycine decarboxylase reaction[a]

| Condition | Glycine decarboxylase activity (U/mg × $10^{-3}$ [%]) in: | |
|---|---|---|
| | Fresh crude extract | Dialyzed crude extract |
| Complete | 28.6 (100) | 22.3 (100) |
| Without dithioerythreitol | 28.4 (99) | 18.0 (82) |
| Without pyridoxal phosphate | 27.0 (94) | 14.5 (65) |
| Without $H_4$-folate | 21.6 (75) | 0 (0) |
| Without NADP | 8.4 (29) | 0 (0) |

[a] The glycine decarboxylase activity was measured in freshly prepared or dialyzed (against 50 mM potassium phosphate buffer, pH 7.8) crude extract by the [1-$^{14}$C]glycine decarboxylation reaction as described in Materials and Methods.

TABLE 3. Enzyme and coenzyme requirements for $^{14}CO_2$ release from [1-$^{14}$C]glycine and for [1-$^{14}$C]glycine synthesis from $NaH^{14}CO_3$

| Condition[a] | Amt of $^{14}CO_2$ produced from [1-$^{14}$C]glycine (nmol/min) | Amt of [1-$^{14}$C]glycine produced from $NaH^{14}CO_3$ (nmol/min) |
|---|---|---|
| Complete + NADP(H) | 106 | 8.4 |
| Without P1 | 0 | 0 |
| Without P2 | 0 | 0 |
| Without P3 | 0 | 0 |
| Without P4 | 0 | 0 |
| Complete + NAD(H) | 24 | 2.1 |
| Without P3 (with diaphorase[b]) | 162 | 12.8 |

[a] Except for the variations indicated, the enzyme assay contained (in 1.5 ml) 75 μg of P1, 100 μg of P2, 50 μg of P3, and 75 μg of P4. Reactions were performed as described in Materials and Methods.
[b] 0.4 U of pig heart diaphorase was used.

homogeneous protein exhibited a $M_r$ of 15,500 as determined by SDS-polyacrylamide electrophoresis and gel filtration on Sephadex G-100. No significant loss of protein P2 activity, as determined by its involvement in glycine bicarbonate exchange reaction, could be measured after heating the protein at 90°C for 10 min.

The transferase protein P4 was purified 20-fold from 30 to 50% saturated ammonium sulfate precipitation (Table 1). Because protein P4 activity was only to be measured by a complementation test with protein P1, P2 and diaphorase, or P3, the P4 activity itself could not be determined in crude extracts. Due to its instability, we did not succeed in purifying protein P4 to homogeneity, for one minor protein band (about 10%) with a larger $M_r$ could be detected in 10% SDS-polyacrylamide gels beside the protein P4 band. The protein P4 showed an $M_r$ of 42,000 by SDS-polyacrylamide gel electrophoresis and an $M_r$ of 45,000 by gel chromatography on Sephadex G-100. The spectra recorded for protein P2 (maxima at 223 and 273 nm) and protein P4 (maxima at 232 and 277 nm and a small shoulder at 305 nm) revealed only the typical absorbance of colorless proteins.

**Determination of the lipoic acid content of protein P2.** The lipoic acid content of protein P2 was determined by using an *E. coli* mutant, which requires lipoic acid for growth (11). To exclude revertants, single colonies were first tested on agar plates for their growth dependence on lipoic acid. Three of four test cultures supplemented with lipoic acid from protein P2 (0.48 μg per test tube) grew up to an optical density which represented 4.3 ng of lipoic acid on a calibration curve. From these results a lipoic acid content of 0.66 mol/mol of P2 was calculated. As a control, tests were run also with the acidic and heat-stable selenoprotein ($M_r$, 13,000) involved in the glycine reductase complex of *E. acidaminophilum* (M. Rieth, Ph.D. thesis, University of Göttingen, Federal Republic of Germany, 1987). Only one of four cultures grew up to an optical density representing 0.7 ng of lipoic acid per 0.5 μg of selenoprotein, which would mean a ratio of 0.09 mol of lipoic acid per mol of selenoprotein.

**Catalytic requirements.** The glycine decarboxylase of *E. acidaminophilum* required NAD(P), $H_4$-folate, pyridoxal phosphate, and DTE to exhibit maximum activity (Table 2), as was known from other glycine cleavage systems (10, 13, 21). All four isolated proteins were necessary to measure overall glycine decarboxylase or synthase activity in the test systems with the radioactive or the optical test, in contrast to glycine synthase from *Arthrobacter globiformis*, which works with NADH and DTE instead of lipoamide dehydrogenase (21). Cell extracts or isolated glycine decarboxylase proteins of *E. acidaminophilus* exhibited highest activities when NADP(H) was used as the electron acceptor or donor, respectively. If protein P3 was replaced by 0.4 U of porcine heart lipoamide dehydrogenase, the activities increased about 1.5-fold with NAD compared with samples without porcine heart lipoamide dehydrogenase addition and with NADP as the electron acceptor of the glycine decarboxylase reaction. Thus, protein P3 seemed to have a lower affinity for protein P2 than did the eucaryotic lipoamide dehydrogenase, for which the isolated protein P2 served as a good substrate (Table 3). No other protein fraction in cell extracts of *E. acidaminophilum* was able to replace protein P3 in the glycine decarboxylase or glycine synthase reaction.

The activities of glycine synthase varied between 3 and 12% compared with glycine decarboxylase when NADH or NADPH was used, indicating a preference for the degradative direction. Glycine was identified as the reaction product of glycine synthase. Protein P1 was unable to catalyze a measurable glycine decarboxylase reaction in the absence of protein P2, in contrast to the P protein from chicken liver mitochondria (13). Protein P1 from *E. acidaminophilum* specifically required protein P2, which could not be replaced by lipoic acid or lipoamide in catalyzing the bicarbonate-glycine exchange reaction. However, protein P2 could partially be replaced (18%) by the same amount (50 μg) of protein P2 from *Peptostreptococcus glycinophilus* (a gift from H. Lebertz, Göttingen, Federal Republic of Germany), another anaerobic glycine-utilizing bacterium. Protein P1 was very specific for protein P2, as documented by a low $K_m$ value of 2 μM (27 μg) for protein P2, determined at constant protein P1 and bicarbonate concentrations (Fig. 2).

The glycine decarboxylase activities of *E. acidaminophilum* measured in crude extracts or with isolated proteins were unexpectedly low when the short doubling time of 60 min on glycine was taken into account. Thus, permeabilized cells were prepared to simulate in vivo conditions. *E. acidaminophilum* cells from the logarithmic growth phase were suspended in anaerobe P1-buffer. One part was frozen in the presence of 0.05% Triton X-100 at −20°C and thawed at 4°C to permeabilize the cells. The other part was used to prepare the regular crude extract as a control. The permeabilized cells showed a glycine decarboxylase activity four times higher than that of the crude extract. Ultracentrifugation of the crude extract (100,000 × *g* for 1 h) did not furnish evidence for a close membrane association of the glycine decarboxylase, for 78% of the activity was found in the supernatant.

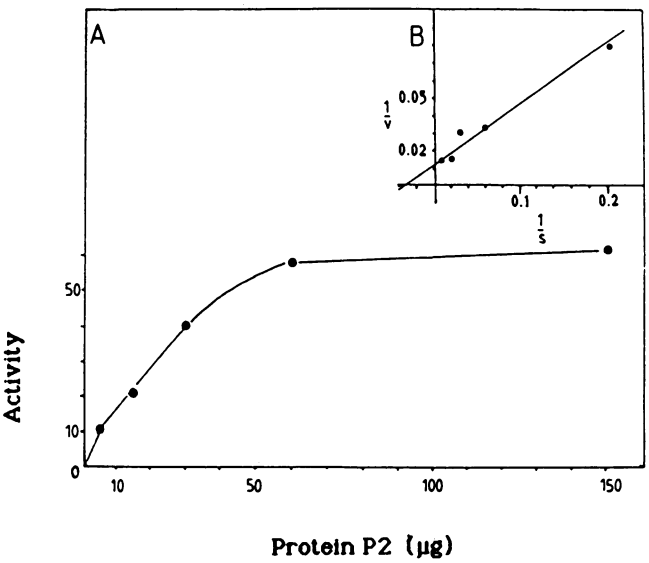


FIG. 2. Determination of the Michaelis constant ($K_m$) of protein P1 for protein P2. The enzyme activity of protein P1 (75 µg) was determined at constant glycine and bicarbonate concentrations as described in Materials and Methods. (A) Rate of enzyme activity ($V$) at various amounts of P2 ($S$). (B) $K_m$ values obtained by the reciprocal of the plot shown in panel A.

**Inhibitors of the glycine decarboxylase reaction.** To further characterize glycine decarboxylase from *E. acidaminophilum*, some potential inhibitors were tested, as were analogs of the substrate or intermediates and metal ions which are known to inhibit the glycine decarboxylase reaction (14, 17). Arsenic trioxide was a potent inhibitor of the glycine decarboxylase (Table 4), probably by reaction with the dithiols of protein P2. Cyanide and azide were also inhibitory at low concentrations. Methylamine can be a product of glycine decarboxylase from chicken liver when the reaction is catalyzed in the absence of H protein (12); however, methylamine was a strong inhibitor of glycine decarboxylase from *E. acidaminophilum*, as indicated by a low $K_i$ value of 0.45 mM determined at constant glycine concentration (10 mM). Thus, glycine decarboxylase of *E. acidaminophilum* was 62-fold more sensitive to methylamine than was glycine decarboxylase from chicken liver (12). Metal ions such as $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Hg^{2+}$ inhibited nearby completely the overall reaction of glycine decarboxylase from *E. acidaminophilum* (Table 4).

TABLE 4. Influence of some inhibitory compounds on glycine decarboxylase activity

| Compound | Concentration in test (mM) | Activity (%)[a] |
|---|---|---|
| Avidin | 1.2[b] | 82 |
| Acetamide | 1.0 | 100 |
| Methylamine | 1.0 | 85 |
| | 5.0 | 41 |
| Glycinonitrile | 1.0 | 90 |
| | 5.0 | 56 |
| $NaN_3$ | 0.1 | 38 |
| KCN | 0.1 | 30 |
| $As_2O_3$ | 0.1 | 6 |
| $MgCl_2$ | 1.0 | 84 |
| $MnCl_2$ | 1.0 | 96 |
| $HgCl_2$ | 0.1 | 5 |
| $FeCl_2$ | 1.0 | 25 |
| $NiCl_2$ | 1.0 | 6 |
| $CoCl_2$ | 1.0 | 7 |
| $ZnCl_2$ | 1.0 | 1 |
| $CuCl_2$ | 1.0 | 7 |

[a] Glycine decarboxylase activity was measured in crude extracts by determine the decarboxylation of [1-$^{14}$C]glycine as described in Materials and Methods. Glycine decarboxylase activities were expressed as a percentage of a control experiment (100% = 24 mU/mg).

[b] Value for avidin is in units; 1 U of avidin binds 1 µg of D-biotin.

**Immunological studies.** The antibodies prepared against proteins P1, P2, and P3 only reacted with the corresponding protein and showed no cross-reaction among the other glycine decarboxylase proteins from *E. acidaminophilum*. Cell extracts from various anaerobic and aerobic microorganisms, most of them glycine-utilizing bacteria, were tested for cross-reactivity in Ouchterlony double immunodiffusion tests. Cross-reactions could only be detected with extracts from *Clostridium cylindrosporum*, organism W6, and *E. angustum* with anti-P1 IgGs, and with extracts from *C. cyclindrosporum* and organism W6 with anti-P2 IgGs, respectively. Anti-P3-IgGs reacted with extracts from the *Clostridium* species *C. cylindrosporum*, *C. sporogenes*, *C. sticklandii*, and organism W6 (5). When anti-P1 and anti-P2 IgGs were used, no cross-reaction was obtained with extracts from the *Clostridium* species *C. acidiurici*, *C. purinolyticum*, *C. sporogenes*, *C. sticklandii*, the peptostreptococci *P. barnesae*, *P. glycinophilus*, *P. parvulus*, *P. prevotii*, *P. variabilis*, Hare strain III and IV, *Acetobacteroides glycinophilus*, *Acidaminobacter hydrogenoformans*, and the aerobic organisms *Arthrobacter globiformis* and *Pseudomonas putida*. The amount of cross-reacting material in extracts of *E. acidaminophilum* could be determined by rocket immunoelectrophoresis, and for anti-P1-antibodies was found to be 3.1% of the soluble protein content and for anti-P2-antibodies was found to be 3.5% of the soluble protein content. For anti-P3-antibodies, the amount of cross-reacting material was determined to be 1.4% (5).

## DISCUSSION

In this paper, we have reported the purification and partial characterization of the glycine decarboxylase complex from *E. acidaminophilum*. Our results indicate that this enzyme complex is composed of four proteins, as previously characterized (7, 8, 12, 16–19, 21, 28, 31, 33, 35); however, the individual proteins were found to have both similar and dissimilar properties. The absorbance spectrum of protein P1 was due to the loosely bound pyridoxal phosphate-containing moiety. Its maximum was at 400 nm, as was observed for the corresponding protein of *C. acidiurici* (9), whereas it was at 430 and 428 nm for the proteins from *P. glycinophilus* (18) and eucaryotic systems (16), respectively, compared with 388 nm of free pyridoxal phosphate. The protein P1 of *E. acidaminophilum* was an $\alpha_2\beta_2$ tetramer, showed an $M_r$ of 225,000, did not contain firmly bound pyridoxal phosphate, and could not be reactivated. These properties were found to be similar to those of the corresponding enzyme (HLC protein) from *C. acidiurici* (9), whereas P protein from chicken liver exists as a homodimer ($M_r$ = 200,000), with each subunit containing equimolar bound pyridoxal phosphate (12). Protein P1 from *P. glycinophilus* is reported to be smaller ($M_r$, 125,000) and contains 2 mol of pyridoxal phosphate per mol of P1 (19). Additionally, purified protein P1 from *E. acidaminophilum* was similar to that from *C. acidiurici* (9) in that it was not able to

catalyze the glycine bicarbonate exchange reaction or glycine decarboxylation in the absence of protein P2, in contrast to the corresponding enzymes from *A. globiformis* (21) and chicken liver (12). Surprisingly, no immunological cross-reaction could be detected with crude extracts of *C. acidiurici* by using anti-P1 or -P2 antibodies, although P1 from *E. acidaminophilum* shared some biochemical similarities with the corresponding enzyme from *C. acidiurici*. However, extracts of *C. cylindrosporum* exhibited cross-reactions, thus further indicating the validity of separating *C. acidiurici* and *C. cylindrosporum* into two individual species, despite their extensive phenotypical similarities (1).

An $M_r$ of 15,500 could be determined for the heat-stable, lipoic acid-containing, hydrogen carrier protein P2 from *E. acidaminophilum*. This enzyme has been isolated from many sources, and molecular masses have been estimated as ranging from 12,000 in human liver cells (15) and *P. glycinophilus* (31) to 20,000 in *C. acidiurici* (9) and *A. globiformis* (21). The $M_r$s of 14,500 and 16,000 for the H protein (P2) from chicken liver (7) and *P. putida* (33), respectively, come close to the $M_r$ of 15,000 calculated for P2 from *E. acidaminophilum*. Lipoic acid was able to replace P2 (H protein) from *A. globiformis* and eucaryotic glycine decarboxylase systems in vitro (12, 22). In our studies, lipoic acid or lipoamide was not able to replace protein P2, as has also been described for the enzymes catalyzing glycine bicarbonate exchange in *C. acidiurici* (9). The lower molar content of lipoic acid found per mole of protein P2 might be a reason for the discrepancy observed between the 10-fold purification obtained by the activity assay and the protein content of 3.5% as determined by rocket immunoelectrophoresis.

Protein P4 is composed of a single polypeptide chain with a molecular mass of about 45,000. This value is similar to those for T proteins from pea leaf (45,000) and chicken liver mitochondria (41,000) as determined by SDS-gel electrophoresis (30, 35). Protein P4 was very unstable during the enrichment procedure. For example, during the last purification step more than 50% of the protein was removed from the P4 fraction, but the specific acitivity increased by only 3%. The relative instability of protein P4 is also known for the T protein from rat liver mitochondria (28).

The most significant difference among known proteins involved in glycine decarboxylase activity was exhibited by the lipoamide dehydrogenase protein P3 in its low $M_r$ of about 68,000 and its preference of NADP (5). Therefore, glycine decarboxylase from *E. acidaminophilum* showed the highest activity with NADP as the electron acceptor instead of NAD. This could be explained by the fact that all other enzymes from *E. acidaminophilum* which act in glycine oxidation prefer NADP (37), and NADPH was the preferred reduced pyridine nucleotide in the glycine reductase reaction (M. Rieth, Ph.D. thesis).

All four glycine decarboxylase proteins from *E. acidaminophilum* were necessary to catalyze both the glycine decarboxylase and the glycine synthase reaction. In contrast to the situation in *P. putida* (33), the glycine-specific lipoamide dehydrogenase (protein P3) could be replaced by another lipoamide dehydrogenase [from porcine heart, when NAD(H) was employed]. Unexpectedly, the activity was even higher under the latter conditions compared with the native protein P3, the only protein with lipoamide dehydrogenase activity in extracts of *E. acidaminophilus* (5). The lower specific activity detected might reflect a different structural composition of the glycine decarboxylase complex in this organism as evidenced by immunocytochemical studies (W. Freudenberg, F. Mayer, and J. R. Andreesen, submitted for publication). We did not succeed in isolating the glycine decarboxylase proteins from *E. acidaminophilus* as an enzyme complex of all four proteins. The low glycine synthase activities obtained in our test systems (12% of glycine decarboxylase activity) indicated that this enzyme complex acts preferentially in glycine oxidation. This might be a reason why *E. acidaminophilum* is not able to grow autotrophically, although all enzymes postulated to be required (3, 6, 36) are present (37). Due to the absence of serine hydroxymethyltransferase (37) glycine has to be synthesized from $CO_2$, whereas all other cell constituents can be formed by reductive carboxylation reactions starting from acetyl coenzyme A (34).

The activity of glycine decarboxylase in cell extract and of isolated proteins was low, as has been observed for all of the other glycine decarboxylase systems studied so far (16). In our case it was somewhat surprising if one takes into account the short doubling time (60 min) of *E. acidaminophilum* growing on glycine (37). In the logarithmic growth phase, about 0.93 μmol of glycine per min was utilized per mg of dry weight of *E. acidaminophilum*. On the basis of the assumption that 1 mg of cell dry weight contains 0.5 mg of protein, a specific activity for glycine-utilizing enzymes of about 1.86 μmol of glycine utilized per min per mg could be calculated. Because the oxidation of 1 mol of glycine to $CO_2$ and $NH_3$ by glycine decarboxylase delivers the reducing equivalents for the further reduction of 3 mol of glycine by glycine reductase, the specific activity of the glycine decarboxylase complex of *E. acidaminophilum* should be about 0.63 U/mg. Thus, the in vivo activities have to be 10-fold higher to explain the high metabolic flux. It is possible that during the disruption of the cells the functional glycine decarboxylase complex was destroyed, causing the low activity observed. This possibility was supported by the fact that in permeabilized cells of *E. acidaminophilum* the glycine decarboxylase acitvity was fourfold higher than in cell extracts. A comparable effect was observed with cells of the glycine-utilizing *Arthrobacter* P1, which showed glycine decarboxylase activity after permeabilization but not in cell extracts (25).

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