When the growth medium of *Clostridium acidiurici* was supplemented with trace metals, glycine synthase and glycine-CO$_2$ exchange activities in cell-free extracts were found to increase significantly. The glycine-CO$_2$ exchange system was purified and shown to consist of a heat-labile component and a heat-stable component. By gel filtration, heat-labile component had an estimated native $M_r = 230,000$ and contained two subunits of $M_r = 65,000$ and 69,000 on sodium dodecyl sulfate-polyacrylamide gels, indicating an $a_2b_2$ tetramer. Heat-stable component had an estimated $M_r = 20,000$ and could not be replaced by lipoidic acid in reaction mixtures. Pyridoxal phosphate was not bound to either of the purified components but was essential for glycine-CO$_2$ exchange. By spectral analysis, heat-labile component was shown to interact with pyridoxal phosphate and that reductant influence this interaction.

Purinolytic clostridia are a specialized group of anaerobes which utilize purines for carbon, nitrogen, and energy. At present, there are only three known species: *Clostridium acidiurici* (1-6), *Clostridium cylindrosorum* (1-6), and *Clostridium purinolyticum* (6, 7). As illustrated in Fig. 1, purines, such as uric acid, xanthine, and quanine, are initially deaminated and decarboxylated to yield formimino-glycine, which is subsequently converted to glycine and formimino-tetrahydrofuran by glycine formimidotransferase (EC 2.1.2.4). Via the glycine-serine pathway, glycine and formimino-tetrahydrofuran are converted to serine which is then degraded to acetate, CO$_2$, and NH$_3$. (See review by Vogels and Van der Drift (5)). However, formimino-tetrahydrofuran can also be converted to formate, and acetate can be derived directly from glycine by glycine reductase (6, 8, 9). The path of carbon flow depends on both the purinolytic species and the purine being fermented.

In addition to the synthesis of purine-derived fermentation products, Barker et al. (1) and Schulman et al. (10) demonstrated that *C. acidiurici* and *C. cylindrosorum* fix CO$_2$ into both the methyl and carboxyl carbons of acetate. Sagers and Gunsalus (11) had earlier observed glycine synthase-like activity in *C. acidiurici*, and Vogels and Van der Drift (5) and Weber and Wood (12) proposed that glycine synthase (EC 2.1.2.10) played a key role in the synthesis of acetate from CO$_2$. Fig. 2 illustrates how both carbons of acetate can be derived from CO$_2$ and the proposed role for glycine synthase. Glycine synthase, also referred to as the glycine cleavage system, is a multicomponent enzyme system and has been characterized in a number of biological systems, including the aerobic bacterium, *Arthrobacter globiformis* (13-15), the anaerobic glycine fermenter, *Peptococcus glycophilus* (16-19), and vertebrate (20-27) and plant (28-30) mitochondria. These glycine synthase systems are composed of four enzymes: (i) a large molecular weight pyridoxal phosphate-containing glycine decarboxylase, designated P-protein or $P_1$; (ii) a small molecular weight, heat-stable lipoyl protein, designated H-protein or $P_2$; (iii) a lipoyl dehydrogenase, designated L-protein or $P_3$; and (iv) a transferase, designated T-protein or $P_4$. (See Refs. 5, 20, and 27 for reviews.) Collectively, these enzymes catalyze the enclosed reaction in Fig. 2. Furthermore, components $P_1$ and $P_2$ jointly catalyze the following glycine-CO$_2$ exchange reaction: $H_2N-CH_2-CH=CO_2+14C0_2 \rightarrow H_2N-CH_2-\rightarrow 14CO_2+H_2O$. This exchange reaction is used to assay for the $P_1$ and $P_2$ components of glycine synthase.

In these previously characterized prokaryotic and eucaryotic glycine synthase systems, the enzyme appears to function primarily in the degradation rather than synthesis of glycine. However, the proposed role for glycine synthase in the purinolytic clostridia is for the formation of glycine. Considering this apparent physiological difference with the previously characterized glycine synthases, we initiated the present study on the glycine synthase system of *C. acidiurici*.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Trace Metals, Growth, and Enzyme Activities**—When the medium described by Rabinowitiz (31) was supplemented with the trace metals indicated under "Experimental Procedures," cell yields of *C. acidiurici* increased from 0.5 g (wet weight) to 1.0 g/liter. Furthermore, as shown in Table I, the specific activities of glycine synthase and the glycine-CO$_2$ exchange reaction in cell-free extracts were also enhanced significantly.

**Purification of Glycine-CO$_2$ Exchange System**—When cell-free extracts were fractionated with ammonium sulfate followed by gel filtration, no single fraction was found to catalyze either glycine synthase or glycine-CO$_2$ exchange reactions.

The abbreviations used are: HLC, heat-labile component; HSC, heat-stable component; SDS, sodium dodecyl sulfate.

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of acetate from glycine synthase folate is synthesized from CO$_2$ by formate dehydrogenase and aacetate by either glycine reductase (path B).

Alternative pathway for converting glycine to acetate (right solid line) is catalyzed by glycine reductase.

HLC was observed to be stable throughout purification except that the specific activity of HLC did not increase significantly in the final stage of purification. While the reason for this is not known, oxidation of a redox-sensitive site on HLC may be involved. We have noted that when dithioerythritol is omitted from the buffer used during the Bio-Gel-HT step, activity of the purified HLC drops greater than 90% in 48 h at 4 °C. Since Hiraga and Kikuchi (25) earlier observed inhibition of the mitochondrial glycine-CO$_2$ exchange system by potassium ions, HLC may also be affected by the high concentration of potassium phosphate used during Bio-Gel-HT chromatography. However, dialysis of purified HLC against 50 mM potassium phosphate, pH 7.0, with 1 mM dithioerythritol failed to significantly enhance activity.

As seen in Fig. 3, the HLC from Bio-Gel HT was electrophoretically pure. In contrast, HLC from Whatman DE52 contained five protein bands on nondenaturing polyacrylamide gels. The possibility that one of these proteins was required for maximal HLC activity was addressed by supplementing reaction mixtures of purified HLC and HSC with the other Bio-Gel HT protein factors; no stimulation was observed. However, we cannot exclude the possibility that an essential factor was at least partially lost from HLC during the final purification step.

HSC from Sephadex G-50 contained two major and three minor protein bands on nondenaturing polyacrylamide gels (data not shown), and efforts to further purify HSC to electrophoretic purity were unsuccessful. Neither HLC nor HSC was purified using strict anaerobic procedures as preliminary tests indicated that glycine synthase and the glycine-CO$_2$ exchange activity were not significantly affected by aeration provided fresh dithioerythritol was added to all purification buffers. If glycine synthase exists as an enzyme complex (20), fractionation of the complex may render its various parts more labile to aeration. Although the mitochondrial and bacterial glycine synthases previously investigated appear fairly similar, the mitochondrial enzyme of C. acidiurici was likely a multicomponent system. We elected to purify the components required for glycine-CO$_2$ exchange as the first step in characterizing the complete glycine synthase system.

As in other glycine synthase systems, two components were required for glycine-CO$_2$ exchange. One was heat-labile (inactivated after 5 min at 90 °C) and designated heat-labile component, while the other was heat-stable (not inactivated after 5 min at 90 °C) and designated heat-stable component. In these regards, HLC and HSC bear similarity to the P-protein and H-protein, respectively, of other glycine synthases (14, 16, 21, 22). Utilizing crude HSC to assay for HLC, HLC was purified first; HSC was subsequently purified by assay with purified HLC. The procedures for the purification of HLC and HSC are described under "Experimental Procedures," and Table II outlines the results obtained from a typical purification.

HSC was purified first; HSC was subsequently purified by assay with purified HLC. The procedures for the purification of HLC and HSC are described under "Experimental Procedures," and Table II outlines the results obtained from a typical purification.

Table I

| Component          | Purification stage | Amount (units/mg) | Total units | Recovery % |
|--------------------|--------------------|-------------------|-------------|------------|
| HLC*               | Cell extract (1550 mg) | 195              | 302         | 100        |
|                    | Ammonium sulfate   | 379              | 151         | 50         |
|                    | Sephadex G-100      | 2467             | 131         | 43         |
|                    | Whatman DE52       | 5250             | 74          | 25         |
|                    | Bio-Gel HT         | 5651             | 18          | 6          |
| HSC*               | Crude HSC (100 mg) | 80               | 8           | 100        |
|                    | Ammonium sulfate   | 115              | 4           | 50         |
|                    | Sephadex G-50      | 195              | 1           | 9          |

*240 units of crude HSC were utilized per assay to quantitate HLC. Control reactions from which HLC fractions were deleted contained less than 1 unit of activity/mg of crude HSC.

*10 units of purified HLC were utilized per assay to quantitate HSC. Control reactions from which HSC fractions were deleted contained less than 1 unit of activity/mg of purified HLC.
stable during purification, the enzyme of *C. acidurici* may be more labile to fractionation.

**Catalytic Requirements and Molecular Weights of the Purified System**—We observed that the ratio of HLC and HSC had dramatic effects on the glycine-CO₂ exchange activity. While optimum activity was observed with a ratio of approximately 1 unit of HLC to 1 unit of HSC, greatly increasing or decreasing the HLC:HSC ratio had negative effects on the glycine-CO₂ exchange activity. The pH optimum for glycine-CO₂ exchange was 7.0, and pyridoxal phosphate was essential to catalysis since a greater than 90% loss in activity was observed when it was deleted from assay mixtures. Deletion of EDTA only resulted in 10% loss in activity and was not considered essential.

With the purified glycine-CO₂ exchange systems from *A. globiformis* (15) and chicken liver mitochondria (24), the heat-stable, lipoyl-containing component, H-protein, could be replaced with lipoic acid. However, no activity was observed when 120 μmol of lipoic acid were substituted for HSC. Additionally, although active in glycine-CO₂ exchange, the purified HLC/HSC system did not contain glycine synthase activity, indicating the requirement for additional components.

The native molecular weights of HLC and HSC, as determined by gel filtration, were estimated to be 230,000 and 20,000 respectively (Fig. 4). SDS-polyacrylamide gel electrophoresis of HLC revealed two bands of apparent equal intensity (Fig. 3), the molecular weights of which were estimated as 65,000 and 58,000 (Fig. 4). This indicates that HLC is a tetramer of α₂β₂ composition.

**Spectral Analyses of HLC**—Pyridoxal phosphate displays an absorption maximum of 390 nm, and when bound to a protein, this maximum shifts to between 420 and 430 nm (44, 45). In other glycine synthase systems, the presence of bound pyridoxal phosphate to P-protein has been indicated by an absorbance maximum around 428-430 nm (17, 18, 24, 26). With the P-protein purified from *P. glyciphilus*, the 430 nm peak increases in absorption when supplemental pyridoxal phosphate is added (17). As shown in Fig. 5, HLC did not display an absorption maxima in the visible region, indicating that pyridoxal phosphate was not bound to the purified protein. However, when dialyzed against buffer containing pyridoxal phosphate, an absorption peak was observed at 401 nm; this peak shifted to 424 nm when dialysis was against buffer containing both pyridoxal phosphate and dithioerythritol (Fig. 5). This absorption peak is evidence of an interaction of HLC with pyridoxal phosphate, and that reductant (dithio-
erythritol) influences this apoenzyme/cofactor interaction. Since HLC appeared to decrease in activity in the last purification step, it seemed possible that the loss in activity may have been due to loss of pyridoxal phosphate from the enzyme. However, no pyridoxal phosphate absorbance maximum was observed in the HLC obtained from Whatman DE52. These observations indicate that, upon purification, HLC does not contain bound pyridoxal phosphate as do other glycine synthase P-proteins. Additionally, no absorption maxima were observed in the visible spectrum of HSC.

**DISCUSSION**

In this study, we have purified from *C. acidiurici* the two components of glycine synthase which catalyze the glycine-CO₂ exchange reaction. This is the first such study with a purine-fermenting organism. The two components, HLC and HSC, were found to have both similar and dissimilar properties to the glycine-CO₂ exchange systems previously characterized. The most apparent similarities are that two components are required for glycine-CO₂ exchange, and that one component, HLC, has a high molecular weight and is heat-labile (analogous to P-protein), while the other, HSC, is smaller and heat-stable (analogous to H-protein).

HLC is an α₂β₂ tetramer of *Mₐ = 230,000* and does not contain bound pyridoxal phosphate when purified. Chicken liver mitochondrial P-protein has a *Mₐ = 200,000 but is an α₂ dimer with each subunit containing equimolar bound pyridoxyl phosphate (24). A similar *Mₐ* was obtained for P-protein from rat liver mitochondria (23). P-protein from *P. gingivalis* has a native *Mₐ = 125,000* and contains 2 mol of pyridoxyl phosphate/mol of P-protein (18), to the best of our knowledge, its subunit composition has not been reported.

H-protein has been characterized from various sources, and the following molecular weights have been observed: human liver, 12,000 (dimer of 23,600) (46); chicken liver mitochondria, 14,500 (24); *A. globiformis, 20,000* (14); *P. gingivalis, 12,600* (tetramer of 48,000) (19). Lipoic acid has been found to be a constituent of H-protein and can replace H-protein in vitro (15, 24). Significantly, in a nonketogenic hyperglycinemic patient, H-protein was devoid of lipoic acid (46). In our study, HSC had a native *Mₐ = 20,000*, and could not be replaced by lipoic acid. The nature of this component of the glycine synthase of *C. acidiurici* will require further purification of lipoic acid. The nature of this component of the glycine synthase of *C. acidiurici* will require further purification of lipoic acid. The nature of this component of the glycine synthase of *C. acidiurici* will require further purification of lipoic acid.

The reaction catalyzed by glycine synthase is reversible, and the direction of catalysis is apparently characteristic of the biological system from which the enzyme is studied. Synthesis of glycine via glycine synthase in vertebrates is not synonymous with the direction of catalysis. This is the first such study with a purine-fermenting organism. The two components, HLC and HSC did not reconstitute glycine synthase, the additional proteins and/or cofactors of the enzyme must also be purified for complete characterization of the purinolytic glycine synthase.

Recent studies by Dürre and Andreesen (6, 8) suggest that with selenium-grown purinolytic clostridia, acetate is formed mostly by glycine reductase rather than the glycine-serine pathway (Fig. 1). Energetically, glycine reductase may be a more favorable route of acetate synthesis. While this would not alter the role of glycine synthase in the formation of glycine from CO₂, it would have marked effects on the labeling pattern of acetate formed from [³⁴C]methylene-tetrahydrofolate and CO₂. If the glycine formed by glycine synthase were converted to acetate by glycine reductase (Fig. 2, path A), only the methyl carbon of acetate would be labeled [¹⁴C]methylene-tetrahydrofolate. Alternatively, if the glycine-serine pathway was operative (Fig. 2, path B), both carbons of acetate would be labeled. Waber and Wood (12) found that *C. acidiurici* formed double-labeled acetate from [¹⁴C]methylene-tetrahydrofolate, thus supporting the glycine-serine pathway as the path of carbon flow in this organism. However, in their study, *C. acidiurici* was not cultivated on a medium enriched with supplemental trace metals, and this may have favored the glycine-serine pathway.

We observed significant increases in cell yield, glycine synthase, and glycine-CO₂ exchange activity when *C. acidiurici* was cultivated on a medium enriched in trace metals. Although the path of carbon flow to acetate was not determined, it is obvious that trace metals have profound physiological effects on *C. acidiurici*. Indeed, *C. acidiurici* and *C. cyclindraspernum* can be differentiated on the basis of their metal requirements for the formation of formate dehydrogenase (49). Additionally, under conditions of selenium starvation, *C. purinolyticum* degrades uric acid to acetate, formate, glycine, NH₃, and CO₂ by an as yet unknown pathway which apparently does not involve either the glycine-serine pathway or glycine reductase (50).

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Glycine Synthase of the Portalvoi Bacillus, Electrophoretic Analysis

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EXPERIMENTAL PROCEDURES

Extraction: C. acidurici, obtained from J. C. Batchelor, Washington University, was harvested in late log phase and stored at -20°C. Cells (10 ml) were harvested in late log phase and stored at -20°C. The cells were suspended in 10 ml 1% Na2SO4, 0.1 M SeO4, 0.1 M MnSO4·H2O, 0.82 M NH4Cl, 0.05 M tris-Cl, 0.05 M tris-Cl, and 0.05 M tris-Cl, and centrifuged. The supernatant was autoclaved and stored at -20°C.

Ion Exchange: Glycine synthase was assayed according to Kuchi [23] and reactivity was assayed in a final volume of 1 ml. A 10% (v/v) assay mixture containing 100 µM PVP, 100 µM phosphate, 1 ml 0.1 M tris-Cl, 0.1 M NaCl, and 0.01 M Tris-Cl, was added. The reaction was started by the addition of 2 units of glycine. The enzyme was assayed at 24°C. The pH was maintained at 7.4 by the addition of 0.1 M NaOH.

Preparation of the enzyme: The enzyme was purified by DEAE-cellulose chromatography. The enzyme was applied to a DEAE-cellulose column (5 x 9 cm) and eluted with a linear gradient of 0.05 M to 0.5 M NaCl. The enzyme was eluted at 0.2 M NaCl.

Purification of the enzyme: The enzyme was purified by ion-exchange chromatography. The enzyme was applied to a DEAE-cellulose column (5 x 9 cm) and eluted with a linear gradient of 0.05 M to 0.5 M NaCl.

Glycine Synthase of C. acidurici

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