Defective glycine cleavage system in nonketotic hyperglycinemia. Occurrence of a less active glycine decarboxylase and an abnormal aminomethyl carrier protein.

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Research Article

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Defective Glycine Cleavage System in Nonketotic Hyperglycinemia

OCCURRENCE OF A LESS ACTIVE GLYCINE DECARBOXYLASE AND AN ABNORMAL AMINOMETHYL CARRIER PROTEIN

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ABSTRACT The activities of the glycine cleavage system in the liver and brain of a patient with nonketotic hyperglycinemia were extremely low as compared with those of control human liver and brain. The activities of glycine decarboxylase (P-protein) and the aminomethyl carrier protein (H-protein), two of the four protein components of the glycine cleavage system, were considerably reduced in both the liver and brain; the extent of reduction was greater in the H-protein. The activity of the T-protein was normal. Purified H-protein from the patient did not react with lipoamide dehydrogenase, and titration of thiol groups with [2,3-^{14}C]*N*-ethylmaleimide suggested that this H-protein is devoid of lipoic acid. This structural abnormality in the H-protein is considered to constitute the primary molecular lesion in this patient with nonketotic hyperglycinemia.

Immunochemical studies using an antibody specific for P-protein showed that the observed reduction in the activity of the P-protein in the patient was due to reduction of the catalytic activity of the protein rather than a decrease in the actual amount of the P-protein. Partial inactivation of P-protein could result secondarily from impaired metabolism of glycine resulting from deficiency in the activity of H-protein. However, the H-protein from the patient could stimulate the P-protein catalyzed exchange of the carboxyl carbon of glycine with $^{14}CO_2$, although the specific activity of the purified H-protein from the patient was only 4% of that of control human H-protein. The content of H-protein in the liver of the patient was ~35% of that of control human liver.

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INTRODUCTION

Hyperglycinemia occurs when there is decreased catabolism of glycine as a result of reduced activity of the glycine cleavage system (1–4). This system has been shown to constitute the major pathway for the catabolism of glycine and serine in vertebrates (5, 6). The system, which is confined to the mitochondria, is composed of four protein components (6, 7): P-protein (a pyridoxal phosphate-dependent glycine decarboxylase);[1] H-protein (a lipoic acid-containing protein, originally referred to as the hydrogen carrier protein and now verified to be the aminomethyl carrier protein) [8]; T-protein (a tetrahydrofolate-requiring enzyme); and L-protein (a lipoamide dehydrogenase). The glycine cleavage reaction is reversible. A schematic representation of the overall reaction is illustrated in Fig. 1. An important feature is that P-protein requires H-protein to catalyze significant decarboxylation of glycine. In the presence of H-protein the decarboxylation of glycine is coupled with reduction of the lipoic acid moiety of H-protein, and the aminomethyl moiety of glycine is immediately bound to the reduced lipoic acid. Furthermore, the combined P-protein and H-protein actively catalyze the exchange of the carboxyl carbon of glycine with CO_2.

Patients with hyperglycinemia have been divided into two general categories referred to as ketotic and nonketotic hyperglycinemia. Ketotic hyperglycinemia

[1] *Abbreviations used in this paper*: DTNB, 5,5′-dithiobis-[2-nitrobenzoic acid]; H-protein, lipoic acid-containing protein; L-protein, lipoamide dehydrogenase; NEM, *N*-ethylmaleimide; P-protein, pyridoxal phosphate-dependent glycine decarboxylase; SDS, sodium dodecylsulfate; T-protein, tetrahydrofolate-requiring enzyme.

is a syndrome that accompanies a number of disorders in which organic acidemia results from deficiency of enzymes involved in the metabolism of branched-chain amino acids (9–11). In nonketotic hyperglycinemia there is no accumulation of organic acids in body fluids (2, 4). Motokawa et al. (3) have reported that in the liver of the patient with the ketotic hyperglycinemia syndrome the content of the entire system, including P-protein, H-protein, and T-protein, may be reduced, suggesting that organic acid metabolites accumulating in the liver may have acted to reduce the synthesis or assembly of the system. Recently, Jaeken et al. (12) have reported that treatment with the anti-epileptic drug, dipropylacetic acid, which is a branched-chain fatty acid, occasionally led to elevation of the concentration of glycine in plasma; and Kochi et al. (13) found that the administration of dipropylacetic acid to rats resulted in a significant reduction of the level of the glycine cleavage system in the liver. These observations are consistent with the concept that the hyperglycinemia in the ketotic hyperglycinemia is a secondary metabolic consequence of the primary disorder in branched-chain amino acid metabolism.

On the other hand, the molecular nature of the glycine cleavage system in nonketotic hyperglycinemia has not previously been studied with methodology currently available. Perry et al. (14) suggested that the defect in a patient with nonketotic hyperglycinemia involved at least the H-protein, although they were unable to detect activity of P-protein in brain. We have examined the glycine cleavage system in liver and brain obtained from a patient who died of nonketotic hyperglycinemia. We have found that the activities of the P-protein and the H-protein were significantly lower than in control tissues. The data indicated that H-protein purified from the patient liver was devoid of lipoic acid.

METHODS

Chemicals. [^{14}C]Bicarbonate was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). [1-^{14}C]Glycine was purchased from New England Nuclear (Boston, Mass.) and used after purification on a column of Dowex (Dow Chemical Co., Midland, Mich.) 50 [H^+] to remove radioactive contaminants. [2,3-^{14}C]*N*-ethylmaleimide ([^{14}C]NEM) was purchased from the Radiochemical Centre (Amersham, England); NAD^+ and NADH, from Oriental Yeast Company (Tokyo); pyridoxal 5′-phosphate, from Kyowa Hakko Kogyo (Tokyo); dithiothreitol, lipoamide and cytochrome *c*, from Sigma Chemical Co. (St. Louis, Mo.) leupeptin (a microbial protease inhibitor), from Protein Research Foundation (Minomo, Osaka, Japan). Tetrahydrofolate was prepared from folic acid by the method of Kisliuk (15), and methylene tetrahydrofolate was prepared chemically from tetrahydrofolate and formaldehyde (16). Other chemicals were obtained commercially.

Case. L.S. died at 15 mo of age at the University of California San Diego Medical Center. Clinical details and the laboratory findings on which a diagnosis of nonketotic hyperglycinemia was based have been reported elsewhere (17). Her course was unusual in that she presented with a rapidly progressive cerebral degeneration after a period of relatively normal development. There was extensive spongy degeneration of the white matter in the brain.

Preparation of homogenates and sonic extracts of human tissues. Portions of the liver and brain of the patient were delivered by hand to Tohoku University, Sendai, Japan, in a frozen state and stored frozen until studied. Control human livers and brains were obtained at autopsy from patients who had died of congenital heart disease (a 6-mo-old male), a lung tumor (a 53-yr-old male), and sudden heart arrest (a 9 yr-old female) at the Tohoku University Hospital and designated controls A, B, and C, respectively; the specimens were also stored frozen until studied. Frozen tissue was thawed and chopped into small pieces with scissors and then homogenized in a glass pestle homogenizer with 9 or 4 vol of 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 1 mM pyridoxal phosphate, and 10 μg/ml of leupeptin. These were designated as 10 and 20% homogenate, respectively. The homogenates were passed through four layers of gauze and used directly. In some experiments the homogenates were subjected to sonication at 140 W for 3 min, and in some experiments the sonicated homogenates were centrifuged at 105,000 *g* for 60 min to obtain clear tissue extracts.

P-protein and H-protein from chicken liver. These proteins were purified from chicken liver mitochondria using the method of Hiraga and Kikuchi (18).

Preparation of antibody against chicken liver P-protein. P-protein purified to homogeneity from chicken liver mitochondria was used to immunize white rabbits using the standard procedure (19). Antisera were collected by bleeding from the ear on day 7 after the second injection of antigen as a booster, and IgG was prepared by the method of Levy and Sober (20). The antibody thus prepared reacted with the P-protein of human liver in a manner similar to that of the P-protein of chicken liver when examined by Ouchterlony double diffusion analysis.

Assay of enzyme activities The activity of the glycine cleavage reaction was determined essentially by the method of Sato et al. (21). In a final volume of 1 ml the reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7.4), 0.4 μmol of pyridoxal phosphate, 1 μmol of NAD^+, 1 μmol of DL-tetrahydrofolate, 10 μmol of [1-^{14}C]glycine (sp act, 0.05 Ci/mol), and 20% homogenate. The reaction was initiated by the addition of the labeled substrate and carried out for 20 min at 37°C in a Warburg-type flask. The reaction was terminated by the addition of 0.1 ml of 50% trichloroacetic acid, and the $^{14}CO_2$ produced was measured as described (13).

P-protein was assayed by measuring the amount of [^{14}C]-bicarbonate fixed in the carboxyl carbon of glycine by the exchange reaction in the presence of an excess amount of H-protein purified from chicken liver mitochondria, essentially according to the method of Motokawa and Kikuchi (17); experimental conditions were those described previously (18, 22).

H-protein was assayed using the glycine-$^{14}CO_2$ exchange reaction in a manner similar to the assay for P-protein except that the reaction mixture was supplemented with an excess of P-protein purified from chicken liver mitochondria.

T-protein was assayed by measuring the synthesis of glycine from methylene-tetrahydrofolate, NH_4Cl and [^{14}C]bicarbonate in the presence of excess chicken liver P-protein and chicken liver H-protein, essentially according to the method of Motokawa and Kikuchi (23). In a final volume of 1 ml the reaction mixture contained 50 μmol of potassium phosphate buffer (pH 7.4), 10 μmol of NH_4Cl, 1.5 μmol of DL-

methylene-tetrahydrofolate, 10 μmol of dithiothreitol, 0.2 μmol of pyridoxal phosphate, 10 μmol of [^{14}C]bicarbonate (sp act, 0.1 Ci/mol), and the enzyme preparation. The reaction was carried out anaerobically at 37°C for 20 min in a test tube, and the radioactivity of the [^{14}C]glycine formed was determined as described (13).

The activity of lipoamide dehydrogenase was determined spectrophotometrically by measuring the lipoamide-dependent oxidation of NADH (24). The reaction mixture contained 150 μmol of potassium phosphate buffer (pH 7.0), 3.75 μmol of EDTA, 0.3 μmol of NADH, 0.3 μmol of NAD^+, 1.2 μmol of lipoamide, and 10% homogenate (0.1 mg protein), in a final volume of 3 ml. The reaction was carried out at room temperature.

Electrophoresis of H-protein. Electrophoresis of H-protein was conducted according to the method of Davis (25) on a 7.5% polyacrylamide gel column (0.4 × 7 cm, pH 8.3) at a current of 4 mA, or on a 10% polyacrylamide gel column (0.6 × 7 cm) containing 0.1% sodium dodecyl sulfate (SDS) at a current of 7 mA by the method of Weber et al. (26). In the SDS-polyacrylamide gel electrophoresis, H-protein had been pretreated in the following way: 40 μl containing 4–5 μg of H-protein and 10 μl of 5% SDS were mixed in a test tube, and the mixture was heated in a boiling water bath for 5 min. After cooling, the mixture was added with 10 μl of 100 mM dithiothreitol and incubated at 45°C for 30 min under N_2 gas to ensure reduction of disulfides in the protein. Then an excess amount of NEM was added to the mixture to prevent reoxidation of thiol groups. Without the treatment with NEM, the sample often underwent polymerization during the course of electrophoresis, possibly by forming an intermolecular disulfide linkage. The NEM-treated H-protein was subjected directly to electrophoresis.

Estimation of molecular weight of H-protein. The molecular weight of H-protein was determined by gel filtration on a column of Sephadex G-75 (2.5 × 45 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) according to the method of Andrews (27) or by SDS-polyacrylamide gel electrophoresis as described above. Ovalbumin (43,000 mol wt), myoglobin (17,400 mol wt) and cytochrome c (11,700 mol wt) were used as standards.

Protein determination. Protein concentrations were assayed using the biuret method (28) for the crude preparations and by the method of Lowry et al. (29) for the purified preparations, using bovine serum albumin as standard.

RESULTS

Activities of the glycine cleavage system and of its individual components. The overall activity of the glycine cleavage system in the liver of the patient with nonketotic hyperglycinemia was extremely low. Its level was only 2–7% of the activities of control human liver (Table I). The activity in the brain was also significantly lower than that of control individuals.

When the activities of the individual components, P-protein, H-protein, and T-protein were determined (Table I), the activity of P-protein in the liver of the patient was 20–40% of the activity in control human liver, but the extent of the reduction in the activity of the H-protein was considerably greater than that of P-protein. The activity of the T-protein in the patient was not different from the control level. The enzyme designated as L-protein is considered to participate in the glycine cleavage reaction, although it remains to be clarified whether or not a specific lipoamide dehydrogenase is involved. The activity of lipoamide dehydrogenase in the liver of the patient was 87 nmol/mg protein per min, whereas the values obtained in control human liver were 110–150 nmol/mg protein per min.

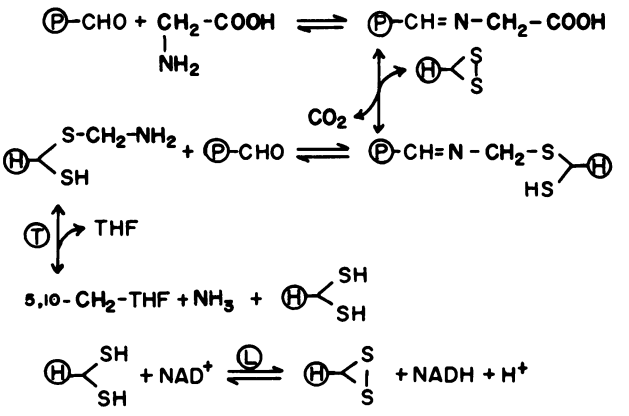

FIGURE 1 The glycine cleavage system. P, H, L, and T circles represent the respective four protein components of the system.

Kinetic properties of P-protein. The P-protein of the patient was found to have an apparent Michaelis constant (K_m) of 4.3 mM for glycine and an apparent K_m of 1.97 μM for the chicken liver H-protein in the

TABLE I

Activities of the Glycine Cleavage System and Its Individual Components in Liver and Brain of a Patient with Nonketotic Hyperglycinemia

Source of tissue	Activity (μmol of product/g tissue/h)			
	Glycine cleavage	P-protein	H-protein	T-protein
Liver				
Patient	0.2	3.5	0.6	5.8
Control A	8.5	16.8	23.7	5.7
Control B	2.6	8.7	8.5	6.0
Control C	3.2	14.2	—	5.0
Brain				
Patient	0.1	0.2	0.7	0.6
Control A	1.2	1.7	5.3	0.4
Control B	0.7	1.2	1.3	1.0

Glycine cleavage activity was assayed using 20% homogenates (5–12 mg protein), and the activities of P-protein, H-protein, and T-protein were assayed using sonicated homogenates (1–4 mg protein). P-protein was assayed in the presence of 100 μg of purified chicken liver H-protein, and H-protein was assayed in the presence of 50 μg of purified chicken liver P-protein. The activity of T-protein was determined by synthesis of the glycine in a reaction system supplemented with 60 μg of chicken liver H-protein and 50 μg of chicken liver P-protein.

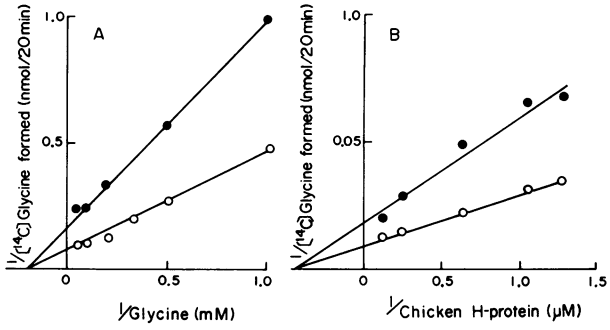

FIGURE 2 Comparison of kinetic properties of P-proteins from the patient and control in the glycine-CO_2 exchange reaction. In A, the reaction mixture contained, in a final volume of 1 ml, 0.2 ml of sonicated liver homogenate of patient or control B as used in the experiments in Table I, 50 μg of purified chicken liver H-protein, and varied amounts of glycine. In B, 0.2 ml of liver homogenate, 10 μmol of glycine, and varied amounts of purified chicken liver H-protein were used. The concentration of H-protein was calculated assuming its molecular weight to be 14,500 (18). ●, patient; ○, control B.

exchange reaction (Fig. 2). These K_m values are in good agreement with the values obtained for control human liver P-protein.

These observations suggest that the primary lesion in this patient is in the reduction of the activity of H-protein and that the reduced activity of the P-protein may be secondary.

Immunochemical analysis of P-protein. To obtain information on whether the observed reduction in the activity of P-protein is a consequence of a decrease in the amount of the enzyme protein, immunotitration of P-protein was carried out using antibody prepared against purified chicken liver P-protein. When extracts of control human livers showing different activities of P-protein were titrated with the antibody, the lines obtained were quite parallel, indicating the P-protein specificity of the antibody. When the extract of the liver of the patient was immunotitrated, the slope of the line was far more gentle as compared with those obtained for control livers, and the amount of the antibody required to precipitate all of the P-protein in the extract was similar to that required for extracts of control human liver. These results suggest that the observed reduction in the activity of P-protein in the liver of the patient does not represent a decrease in the actual amount of enzyme protein but may result from a reduction in the catalytic activity of the P-protein.

Purification and properties of H-protein. Sonic extracts of specimens of liver from the patient and control C were dialyzed overnight against 2 liters of 20 mM potassium phosphate buffer (pH 7.0) and diluted with an equal volume of buffer. The diluted extracts (crude extract) were subjected separately to the purification procedures indicated in Table II.

The crude extracts were heated in a boiling water bath for ~2 min and centrifuged at 13,000 *g* for 20 min. The supernate obtained was applied to a column of DE-32 (2 × 28 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The column was washed with 200 mM NaCl in the same buffer and then H-protein was eluted with a linear gradient of NaCl between 200 and 600 mM in 20 mM potassium phosphate buffer (pH 7.0), and 10-ml fractions were collected. The H-protein activity-containing fractions of eluate in the control liver system were located using the glycine-$^{14}CO_2$ exchange reaction. The H-protein contained in the patient system was recovered in the comparable fractions (Fig. 3). The fractions containing H-protein were combined separately for each series and dialyzed overnight against 2 liters of 20 mM potassium phosphate buffer (pH 7.0). They were designated DE-32 fractions and assayed for the activity of H-protein and for protein concentration.

The DE-32 fraction was concentrated using a hydroxyapatite column (1.5 × 2.5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0). H-protein was eluted from the column with a small volume of 100 mM potassium phosphate buffer (pH 7.0), and the eluate (4 ml) was applied to a column of Sephadex G-75

TABLE II
Purification of Human H-Protein from Extracts of Liver from Patient and Control

Fraction	Control H-protein				Patient H-protein			
	Total protein	Total activity	Specific activity	Recovery	Total protein	Total activity	Specific activity	Recovery
	mg	*U*	*U/mg protein*	%	*mg*	*U*	*U/mg protein*	%
Crude extract	1,793	1,445	0.81	100	1,529	25.0	0.017	100
Supernate after heat treatment	107	1,571.8	14.0	108	101	37.8	0.275	109
DE-32 fraction	1.12	945.8	844.4	65	0.62	16.0	25.81	63
Sephadex G-75 fraction	0.26	451.2	1,735.4	31	0.084	7.1	84.52	28
2nd Sephadex G-75 fraction	0.15	319	2,126.7	22	—	—	—	—

Purification was started using 56 ml of crude liver extract in each series of experiments.

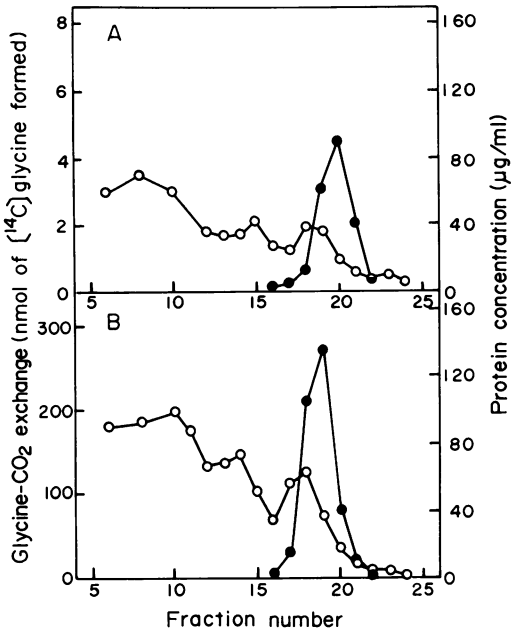

FIGURE 3 Elution profiles of H-protein on DE-32 column chromatography. In A, 101 mg protein of the sample from the liver of the patient and in B, 107 mg protein of the sample from the control human liver were applied to the column. ●, H-protein activity; ○, protein concentration.

(2.5 × 45 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0). H-protein was eluted from the column with the same buffer. At this step of purification, the H-protein of the patient had been purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis (Fig. 4A and C), but the H-protein of the control liver still contained small amounts of contaminating proteins. Therefore, the H-protein fraction of the control liver system was lyophilized and rechromatographed on a column of Sephadex G-75 as above. The H-protein obtained was apparently homogeneous (Fig. 4B and D).

The recovery of H-protein as estimated on the basis of catalytic activity was quite similar in the patient and control systems. This suggests that both H-proteins were purified in quite parallel fashion throughout the purification process. Assuming this, we may estimate from the values for the total protein recovered in the Sephadex G-75 fractions that the content of H-protein in the liver of the patient was ~35% of the content of H-protein in control human liver. However, the value obtained for the specific activity of the H-protein of the patient was only ~4% of the value obtained for the control human H-protein. Consequently, the total activity of H-protein in the liver of the patient has been estimated at <2% of control activity. The values for total activity in Table II are quite consistent with this analysis. These observations suggest that the H-protein of the patient is an abnormal H-protein that exhibits very limited catalytic activity.

The H-protein purified from either the liver of the patient or the control was found to have an apparent molecular weight of ~23,000 when estimated by gel filtration (Fig. 5). However, on SDS-polyacrylamide gel electrophoresis, the H-protein from both the patient or the control human were found in a single band that would correspond to a molecular weight of ~12,000 (Fig. 4C and D). These data suggest that H-protein may have been purified as a homodimer. The H-proteins from the patient and the control migrated quite parallel to each other on polyacrylamide gel electrophoresis in the absence of SDS as well (Fig. 4A and B), indicating that both H-proteins have quite similar electrostatic properties. This is also reflected in the similarity of the elution profiles of the H-proteins on DE-32 column chromatography (Fig. 3).

Examination for lipoic acid. H-protein is known to contain a lipoic acid moiety and to mediate reduction of 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB) in the lipoamide dehydrogenase reaction. This analysis is shown in Fig. 6. The H-protein purified from the liver of the patient displayed no ability to mediate the reduction of DTNB by NADH catalyzed by a purified pig heart lipoamide dehydrogenase. The data obtained were identical to those of the system in which no H-protein was added. In contrast, the H-protein purified from the control human liver was quite active. Its activity in this reaction system was equal to that of purified chicken liver H-protein. These observations suggested that the H-protein from the patient was devoid of lipoic acid.

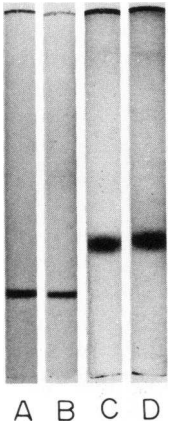

FIGURE 4 Polyacrylamide gel electrophoresis of H-proteins purified from control human liver and the liver of the patient. (A and B) electrophoresis without SDS; (C and D) electrophoresis with SDS. H-protein samples applied were: (A) 4.5 μg from the patient; (B) 3.0 μg from the control; (C) 5.8 μg from the patient; (D) 4.0 μg from the control.

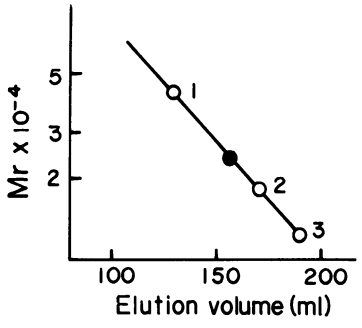

FIGURE 5 Determination of molecular weight of H-protein by Sephadex G-75 column chromatography. Aliquots of 1.1 mg of control human H-protein and 0.6 mg of the H-protein of the patient (DE-32 fraction) were used. ●, H-protein from either source; ○ 1, ovalbumin; ○ 2, myoglobulin; ○ 3, cytochrome *c*.

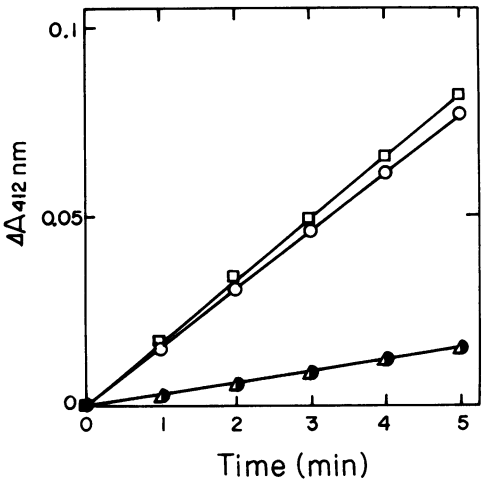

FIGURE 6 Reduction of DTNB mediated by H-protein in the lipoamide dehydrogenase reaction. The reaction was conducted in the cuvette of a recording spectrophotometer. The reaction mixture contained, in a final volume of 1.9 ml, 300 μmol of potassium phosphate buffer (pH 8.0), 2 μmol of EDTA, 10 μg of lipoamide dehydrogenase, 0.5 μmol of NADH, and H-protein. The reaction was initiated by the addition of 0.1 ml of 10 mM DTNB after a 3-min preincubation at 37°C, and the change in absorbance at 412 nm was recorded against a blank that consisted of buffer and DTNB. ●, 5.3 μg of H-protein of the patient; ○, 5.3 μg of control H-protein; □, 5.5 μg of purified chicken liver H-protein; △, H-protein.

To test this hypothesis, we undertook to measure and compare the numbers of thiol groups in the H-proteins purified from the patient and the control using [^{14}C]NEM. The experiment was designed to trap available thiol groups in the reduced form of the H-protein using [^{14}C]NEM. In a final volume of 1 ml, a mixture containing 22 μg of H-protein, 100 μmol of sodium phosphate buffer (pH 7.2), 2 μmol of EDTA, and 1% SDS, was placed in the main compartment of a Warburg-type flask with two arms, and 2 μmol of dithiothreitol and 10 μmol of [^{14}C]NEM were placed in the respective side arms. The gas phase was replaced with N_2 after repeated evacuation and filling, then dithiothreitol was tipped into the main compartment, and the mixture was incubated at 45°C for 45 min. Then [^{14}C]NEM was tipped in, and the mixture was further incubated at 25°C for 30 min. After the incubation, the flask was opened, and 10 μl (equivalent to ~140 μmol) of 2-mercaptoethanol was added to the mixture to nullify the [^{14}C]NEM remaining. The H-protein thus labeled with [^{14}C]NEM was separated using a column of Sephadex G-25 (1.5 × 45 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 0.1% SDS, and examined for radioactivity and protein concentration. The number of [^{14}C]NEM-reactive thiol groups in the oxidized form of H-protein was measured in the same way except that the reduction with dithiothreitol was omitted. The results of these experiments are shown in Fig. 7. The elution profiles for radioactivity and protein were well coordinated in each experiment, indicating that the experimental procedures used were quite reliable.

The molar ratios of protein-bound [^{14}C]NEM to H-protein in individual experiments were calculated. In control human H-protein, the ratio of NEM:H-protein was 0.8 in the oxidized form and 3.6–4.1 in the reduced form. These data indicate that in the reduced state H-protein has a total of four thiol groups, whereas in the oxidized state there is only one thiol group. Two thiol groups in the reduced H-protein may be ascribed to the lipoic acid moiety, and the other two may represent cysteine residues in the protein. In the oxidized state, H-protein may exist as a homodimer possibly resulting from intermolecular disulfide formation, as suggested by the results of gel filtration (Fig. 5).

On the other hand, the H-protein of the patient appeared to have only two thiol groups per molecule in the reduced state. The ratio of NEM:H-protein was 1.6–1.8. The oxidized form appeared to have one thiol group per molecule of H-protein because the ratio was 0.8:1.0 as in control human H-protein. The data are quite compatible with the hypothesis that H-protein of the patient contained no lipoic acid.

We have previously shown that P-protein alone is able to catalyze to a slight degree the exchange of the carboxyl carbon of glycine with CO_2 and that the catalytic activity of P-protein was considerably increased by the addition of free lipoic acid (8, 18). The exchange activity observed in the presence of lipoic acid was far lower (~1,000th) than that observed in the presence of H-protein. Therefore, it was of interest to test the possibility that externally added lipoic acid might function as a cofactor for the H-protein of the patient. If this were the case, the rate of the glycine-

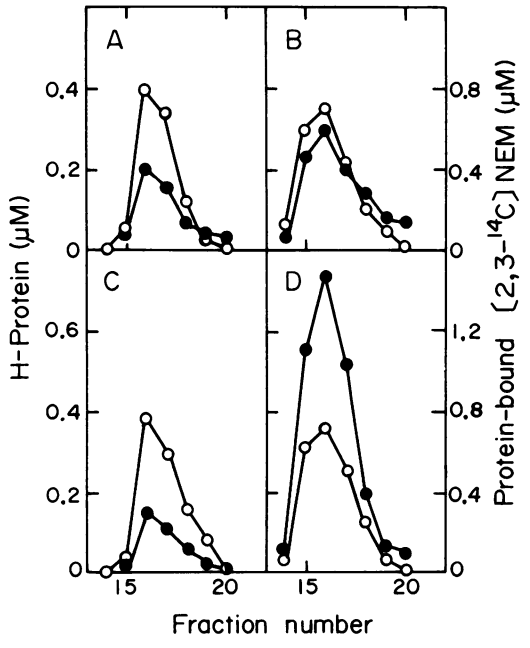

FIGURE 7 Isolation of H-protein labeled with [2,3-^{14}C]NEM by gel filtration using a column of Sephadex G-25. Labeling of H-protein with [^{14}C]NEM was conducted using 22 μg of H-protein in each experiment. (A) H-protein of the patient labeled in the oxidized form; (B) H-protein of the patient labeled in the reduced form; (C) control human H-protein labeled in the oxidized form; (D) control human H-protein labeled in the reduced form. ○, H-protein; ●, protein-bound [^{14}C]NEM.

CO_2 exchange should be greatly increased by the addition of lipoic acid along with the H-protein of the patient. To test this possibility the series of experiments was carried out using purified chicken liver P-protein (Table III). It was found that the exchange reaction was not stimulated. Instead it was appreciably inhibited by the addition of lipoic acid in systems containing H-protein from either the patient or control. These observations suggest that the externally added lipoic acid does not readily bind with H-protein of the patient in a position that permits a role as the functional cofactor of H-protein.

DISCUSSION

In the studied patient with nonketotic hyperglycinemia, an abnormal H-protein in the liver appeared to be devoid of lipoic acid. Although the study was limited to the H-protein of the liver, it seems highly probable that the H-protein in the brain and other tissues was also devoid of lipoic acid because the characteristics of the abnormal glycine cleavage system in the brain were quite similar to those of the liver. This anomaly in the H-protein is considered to constitute the primary lesion in this patient. The absolute activity of H-protein in the brain of the patient was virtually the same in liver and brain, but the activity in control liver was higher than that of control brain (Table I). Thus the relative reduction in the brain may not have been as great. Further studies are needed to clarify whether the H-proteins of the brain or other tissues lack lipoic acid. Perry, et al. (14), had suggested that there was a defect in H-protein in the brain of a patient with nonketotic hyperglycinemia. Our observations represent the first definitive description of the nature of the anomaly in this lipoic acid-containing enzyme. An inherited defect in lipoic acid acetyltransferase has been suspected in a patient with familial intermittent lactic acidosis (30), but the nature of the suspected defect has not been clarified.

In our patient, the activity of the P-protein was also significantly reduced. The observed reduction in the activity of the P-protein did not appear to be due to a decrease in the actual amount of enzyme protein but appeared to reflect a partial inactivation of P-protein presumably as a result of the anomaly in the H-protein. A similar situation has been observed in the case of the P-protein in rats treated with dipropylacetic acid (22), where it was suspected that the P-protein may have been modified by some intermediate(s) of the metabolism of dipropylacetic acid so that it exhibited a lower catalytic activity (13, 22). The P-protein of treated rats had kinetic properties quite similar to those of the P-protein of control rats except for the maximum velocity (V_{max}) as examined in the glycine-CO_2 exchange reaction (22). Similarly, the kinetic properties of the

TABLE III
Effect of Addition of Lipoate on the Glycine-CO_2 Exchange

H-protein	Lipoate	[^{14}C]Glycine formed	
	μM	*nmol*	*%*
None	0	Trace*	
	10	0.1	
	100	0.6	
Patient H-protein	0	2.2	100
	10	1.6	73
	100	1.3	59
Control human H-protein	0	145.9	100
	10	112.7	77
	100	84.7	58

In a final volume of 1 ml, reaction mixtures contained 20 μmol of monobasic potassium phosphate, 20 μmol of $NaH^{14}CO_3$, 1 μmol of dithiothreitol, 0.1 μmol of pyridoxal phosphate, 10 μmol of glycine, 47 μg of purified chicken liver P-protein, indicated concentrations of lipoate, and 1.2 μg (0.1 nmol) of H-protein. Reaction was initiated by the addition of glycine and carried out for 20 min at 37°C.
* Falls within the range of background.

P-protein in our patient with hyperglycinemia were the same as those of the P-protein of control individuals. These observations suggest that the P-protein in the patient may also have been modified in some way, although the molecular mechanism of the modification may not necessarily have been the same as that in the dipropylacetic acid-treated rat. Another possibility that would account for the occurrence of a less active P-protein would be a positive cross-reacting material but inactive mutation in the P-protein. This seems to be unlikely, because it would require genetic mutations in two proteins of the glycine cleavage system in a single patient.

Another possibility is that partial inactivation of the P-protein as suggested by immunotitration could result from prolonged storage of the specimen of liver. To answer this question, we examined the activities of mitochondrial enzymes that are not related to the glycine cleavage system, including glutamate dehydrogenase (a matrix enzyme) (31), monoamine oxidase (an outer membrane enzyme) (32), succinate dehydrogenase (an inner membrane enzyme) (33), and cytochrome oxidase (an inner membrane enzyme) (34), and found that the activities of glutamate dehydrogenase, monoamine oxidase and cytochrome oxidase in mitochondria derived from the liver of the patient were quite similar to those of control human liver mitochondria, whereas the activity of succinate dehydrogenase in the patient was about half of those in controls. Whether the observed reduction of the activity of succinate dehydrogenase in the liver of the patient has any relation to nonketotic hyperglycinemia is obscure until further patients are studied. It may be relevant that Revsin et al. (35), reported a 59% decrease in the activity of propionyl-CoA carboxylase in lysates of fibroblasts derived from a patient with nonketotic hyperglycinemia, and Stumpf et al. (36) showed that propionyl-CoA is a potent inhibitor of succinyl-CoA ligase; half-maximal inhibition occurred at 0.2 mM propionyl-CoA. In any case the observed reduction in the activity of the P-protein does not appear to be an artifact of storage of the liver.

The content of the H-protein in the liver of the patient was reduced to about one-third that of control human liver. The reason for this reduction has not been established, but a reasonable hypothesis is that the abnormal H-protein lacking lipoic acid may be degraded at a higher rate than the normal H-protein. Reduction of the content of H-protein could contribute to the observed low level of activity of glycine cleavage observed in this patient. However, in rat liver the molar concentration of H-protein has been shown to be about nine times greater than that of P-protein (13), suggesting that the liver contains more H-protein than required to meet the physiological needs to the glycine cleavage system.

Even though the H-protein from our patient appeared to have no reactive lipoic acid, the H-protein could still stimulate P-protein-catalyzed exchange of glycine and CO_2. The specific activity of the H-protein of the patient, as estimated by the degree of stimulation of the exchange reaction, however, was only ~4% that of control. In this study we could not carry out analysis of lipoic acid by chromatography or bioassay to confirm its lack in the H-protein because of the absence of additional H-protein for further studies. Therefore, we cannot conclusively exclude the possibility that the H-protein purified from the liver of the patient may have contained ~4% of the amount of intact H-protein containing lipoic acid. However, this possibility seems unlikely in view of the fact that the purified H-protein exhibited no activity in the reduction of DTNB by NADH when incubated with a purified pig heart lipoamide dehydrogenase. Alternatively, the observed 4% activity could represent a conformational change in P-protein brought about by H-protein, which led to catalytic activity on the part of P-protein. This would be independent of the function of lipoic acid. We have made several observations that support the view that a protein-protein interaction between P-protein and H-protein normally induces a conformational change in P-protein that is relevant to the expression of catalytic activity by P-protein (8). In studies with highly purified chicken liver P-protein and H-protein, it was observed that H-protein causes a significant change in the absorption spectrum of P-protein as a pyridoxal phosphate enzyme, and a titration experiment indicated that two molecules of H-protein bind to one molecule of P-protein, or one molecule of H-protein binds to one subunit of P-protein. Furthermore, H-protein acts to reduce the dissociation constant (K_d) of P-protein and methylamine, which is the product of the decarboxylation of glycine catalyzed by P-protein alone. Methylamine inhibits the glycine-CO_2 exchange reaction competitively with glycine. In the presence of H-protein the K_d was reduced from 63 mM to 27 mM, and the K_d value of 27 mM was virtually equal to the inhibition constant (K_i) value for methylamine in the glycine-CO_2 exchange reaction in the presence of H-protein. Finally, P-protein and H-protein form a fairly stable complex that could be demonstrated by gel filtration and by sucrose density gradient centrifugation. On the basis of these and other findings, we have concluded that H-protein plays a dual role in the decarboxylation of glycine. One is as a regulatory protein for P-protein, and the other is as an electron pulling agent and carrier of the aminomethyl moiety derived from glycine by decarboxylation (8). It seems quite possible that the H-protein of the patient also could serve as a modulator of P-protein and in this way stimulate some catalytic activity for P-protein. The H-protein from the patient was shown to have electrostatic properties

quite similar to those of control human H-protein. The fact that an H-protein lacking lipoic acid could stimulate the catalytic activity of P-protein is further evidence for the idea that H-protein functions as a modulator of P-protein.

Inasmuch as the glycine cleavage system is composed of four different proteins, there may be a variety of types of nonketotic hyperglycinemia, each resulting from a defect in a specific component of the glycine cleavage system. The clinical manifestations of the disease could be expected to be quite different in patients with abnormalities in the different individual protein components. Thus, although our patient was found to have an abnormal H-protein, it is highly desirable to carry out detailed investigation of other patients, especially those with different phenotypes, in order to establish the sites of the defect in the glycine cleavage system and to determine their nature.

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