ON THE UTILIZATION OF L-GLUTAMINE BY GLUTAMATE DEHYDROGENASE

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Received March 16,1976

SUMMARY

The action of glutamate dehydrogenase on L-glutamine was followed by determining the formation of α-ketoglutaramate. The rate of the reaction with L-glutamine was about 0.01% of that observed with L-glutamate. The findings suggest that α-ketoglutaramate present in tissues arises mainly by transamination rather than by oxidation of glutamine. Glutamate dehydrogenase does not catalyze glutamate formation from α-ketoglutarate and L-glutamine at a significant rate, but the present findings do not exclude the possibility that glutamine amide nitrogen is used for synthesis of α-amino groups in the mammal by pathways involving coupling between glutamate dehydrogenase and glutaminase (or ω -amidase) or a glutamine-binding subunit, i.e., by reactions equivalent to that catalyzed by glutamate synthase.

α-Ketoglutaramate, the α-keto analog of glutamine (1-8), is present in rat liver, kidney and brain (8), and increased concentrations of α-ketoglutaramate occur in the cerebrospinal fluid in hepatic coma (9). α-Ketoglutaramate was initially investigated as a product of the transamination of glutamine (1-5), and was first prepared by oxidation of L-glutamine with snake venom L-amino acid oxidase (3). Although it is evident that α-ketoglutaramate can be formed in mammalian tissues by transamination of glutamine, other pathways for its formation are conceivable. Recently it was reported that L-glutamine is a good substrate of glutamate dehydrogenase and that it is oxidized (as judged by DPNH formation) by frog and beef liver glutamate dehydrogenases at rates of 36% and 7.4%, respectively, of the rates observed with L-glutamate (10). Such oxidation of L-glutamine would be expected to produce α-ketoglutaramate; this reaction might then contribute to the steady state tissue concentrations of this α-keto acid. We have therefore investigated the action of glutamate dehydrogenase on L-glutamine, and also the possibility that glutamine might, as reported (11), replace ammonia in the glutamate

dehydrogenase-catalyzed synthesis of L-glutamate from α-ketoglutarate; the latter reaction would be analogous to that catalyzed by glutamate synthase (12-15).

MATERIALS AND METHODS: L-Glutamine was obtained from Mann (A), International Mineral and Chemical (B), Sigma (C), Merck (D) and CalBiochem (E). Preparation (F) was purified (5) in 1953 and was stored since then at room temperature. L-Glutamic acid, Na α-ketoglutarate, Na pyruvate, DPN, DPNH, TPN, TPNH, and ATP were obtained from Sigma. Bovine liver glutamate dehydrogenase was obtained as suspensions in 50% $(NH_4)_2SO_4$ and in glycerol from Boehringer-Mannheim, and freed from ammonia by gel filtration on Sephadex G-25; these preparations had the same activity (120 μmoles/min/mg; 25°) and showed a single band on polyacrylamide gel electrophoresis in sodium dodecylsulfate at pH 8.6. Frog liver glutamate dehydrogenase was prepared as described (16). Aerobacter aerogenes glutamate dehydrogenase was obtained as a by-product in the isolation of glutamate synthase (15). Lactate dehydrogenase (rabbit muscle) was obtained from Sigma, and ω-amidase was prepared as described (17). Glutamine synthetase (sheep brain) (18) was kindly provided by Dr. Vaira P. Wellner.

In the studies described in Fig. 1, L-glutamine was purified by passing 5 ml of a 200 mM solution through a Dowex-1-acetate column (0.5 x 5 cm). 5-Oxoproline was determined essentially as described (19). The ammonia content of glutamine solutions was determined by a modification of the Conway procedure (20) in which 0.05 ml of glutamine solution and 0.2 ml of 3.3 M Na_2CO_3 were added to separate regions of vaccine bottles (2.5 x 6 cm); these were closed with a stopper equipped with a glass rod which projected into the bottle and which had been moistened with 0.1 ml of 1.8 M H_2SO_4 just prior to use. The contents were mixed and the bottles were rotated on a wheel for 90 minutes at 26°. The rods were then mixed in 1 ml of Nessler's solution and the colors obtained were compared with those of standards. Under these conditions (pH 10.4), there was complete recovery of added NH_4Cl (0.01-0.1 μmole), and the deamidation of glutamine was less than 0.008%.

RESULTS: Six samples of L-glutamine were tested (Table I); substantial DPNH formation was found with 4 of these, but the activity observed is consistent with the amount of glutamate found by amino acid analysis in the glutamine samples. Two samples (E and F) contained no detectable glutamate and were not active nor were they active when DPN was replaced by TPN. The data show that L-glutamine is less than 0.1% as active as L-glutamate. Similar data were obtained with 2 preparations of frog liver glutamate dehydrogenase.

The formation of α-ketoglutaramate from L-glutamine was studied in mixtures (final vol. 0.2 ml) containing DPN (1 mM), L-glutamine (20 mM; see Methods), 40 mM Na pyruvate, lactate dehydrogenase (200 μg), beef liver glutamate dehydrogenase (1 mg) and ammediol buffer (200 mM; pH 9.0); 37°. Aliquots (10 μl) were removed at intervals and added to Dowex 1-acetate columns (0.5 x 2 cm) and to Dowex 50 (H^+) columns

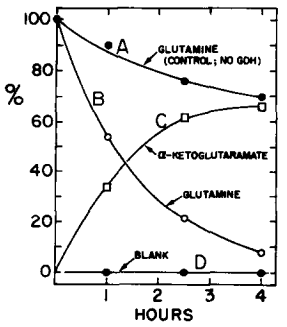

Fig. 1: Formation of α-ketoglutaramate from glutamine catalyzed by bovine liver glutamate dehydrogenase. The experimental details are given in the text. A. Disappearance of glutamine (glutamate dehydrogenase omitted). B. Disappearance of glutamine; complete system. C. Formation of α-ketoglutaramate; complete system. D. Blank; formation of α-ketoglutaramate in the absence of glutamate dehydrogenase or of DPN.

(0.5 x 2 cm). The columns were eluted with 2 ml of water and the effluents were analyzed, respectively, for glutamine (21) and α-ketoglutaramate (8). When ω-amidase was omitted from the latter assay, only traces of α-ketoglutarate were found, i.e., <0.5% of the α-ketoglutaramate. α-Ketoglutaramate was also detected by ascending paper chromatography in a solvent consisting of tert butanol:methylethylketone:formic acid: water (90:30:15:15; v/v); Rf x 100, 75 (5-oxoproline, 82), detected by the modified (22), Rydon and Smith (23) method. About 90% of the glutamine disappeared in 4 hours (Fig. 1,B) with concomitant formation of α-ketoglutaramate (C). In the absence of glutamate dehydrogenase about 30% of the glutamine was converted to 5-oxoproline (A). No α-ketoglutaramate was found in the absence of glutamate dehydrogenase or of DPN (D). The rate of L-glutamine oxidation, based on DPNH formation in mixtures containing ammediol buffer (200 mM; pH 9), L-glutamine (20 mM; see Methods), ADP (0.1 mM),

TABLE I. Activity of Bovine Liver Glutamate Dehydrogenase on L-Glutamine Preparations[+/]

Sample of L-Glutamine (24 mM)	Rate of DPNH Formation[++] nmoles/min	Glutamate in Glutamine Preparation* mM
A	142	0.61
B	122	0.47
C	91.6	0.34
D	86.8	0.32
E	<0.8	<0.01
F	<0.8	<0.01

*By amino acid analysis. [+/] The reaction mixtures contained the enzyme (20 μg), DPN (3.16 mM), L-glutamine preparation (24 mM), and 0.08 M Tris-acetate buffer (pH 8.4); final volume, 0.5 ml; 26°. [++] With L-glutamate concentrations of 1.44, 1.18, 0.75, 0.60, 0.47, 0.36, and 0.29 mM, the respective rates were: 306, 249, 205, 171, 146, 106, and 76.4.

NaEDTA (1 mM), DPN (1 mM) and enzyme (210 μg) at 21°, was 0.25 μmole/hr/mg of enzyme; at pH 8.2, a value of 0.09 was obtained compared to a value of 720 for L-glutamate. The pH optimum for glutamine oxidation (in Tris and ammediol buffers) is 8.8-9.4, compared to pH 8.0-8.4 for glutamate.

We also investigated whether L-glutamine can replace ammonia in the direction of glutamate synthesis under conditions previously used (11), i.e., mixtures containing the bovine liver enzyme, K phosphate (10 mM), Tris (10 mM), Na α-ketoglutarate (14 mM), DPNH (0.16 mM), and L-glutamine (200 mM) at pH 8.3. The rate of DPNH oxidation was 1.65 μmoles/min/mg (3.0 μmoles/min/mg in the presence of 0.5 mM ADP), as compared to 97.3 when glutamine was replaced by NH_4Cl. Studies on the effect of NH_4Cl concentration showed that the presence of about 0.1 mM NH_4^+ could account for the observed activity. Similar results were obtained with TPNH and with A. aerogenes glutamate dehydrogenase. It is well known that glutamine undergoes nonenzymatic conversion to NH_4^+ and 5-oxoproline, and we have found that L-glutamine preparations contain at least

0.1% of 5-oxoproline. In agreement with reported data (11) we found that incubation of bovine liver glutamate dehydrogenase (0.5 mg per ml) in 0.025 M Tris-acetate buffer (pH 8.4) containing 50 mM L-glutamine for 18 hours at 30° did not lead to glutamate formation; there was under these conditions substantial formation of 5-oxoproline in both the presence and absence of the enzyme, a result consistent with earlier data on the non-enzymatic deamidation of glutamine (24). We found (20) 0.35, 0.15, 0.22, 0.24, 0.13, and 0.24% ammonia, respectively, in the glutamine preparations (A $\longrightarrow$ F) used here. Passage through Dowex 1-acetate did not remove ammonia. To remove ammonia, we carried out the glutamate dehydrogenase reaction in the presence of glutamine synthetase, ATP, and Mg^{++}. Whereas oxidation of DPNH was observed when the enzyme was incubated with α-ketoglutarate, DPNH, and glutamine (Table II, exp. 1), no activity was found in the presence of the glutamine synthesis system which has a high affinity for ammonia (exp. 2). When ATP and Mg^{++} were omitted, oxidation of DPNH occurred (exp. 3).

DISCUSSION: Much higher rates of L-glutamine deamination by glutamate dehydrogenases were reported in studies in which the reaction was followed by DPNH formation (10, 25, 26), but in no case was α-ketoglutaramate formation measured, nor was the content of glutamate in the glutamine used reported. In our studies, activity toward L-glutamine, as measured by DPNH formation, correlated well with the glutamate content of the glutamine preparation (Table I). In view of the very low rate of glutamine oxidation we found with purified glutamine, it seems probable that most of the α-ketoglutaramate in tissues arises by transamination rather than by oxidation of L-glutamine by glutamate dehydrogenase.

We also found very low rates of reduced pyridine nucleotide oxidation when glutamine was substituted for ammonia in the direction of reductive amination, and that the small amount of ammonia in the L-glutamine preparation would be sufficient to account for the results.

TABLE II. Experiments in the Presence of Glutamine Synthetase

Experiment	Rate of DPNH oxidation ΔA 340 nm/min
1. α-Ketoglutarate + glutamine	0.064
2. α-Ketoglutarate + glutamine + glutamine synthesis system	<0.01
3. α-Ketoglutarate + glutamine + glutamine synthesis system lacking ATP and $MgCl_2$	0.079
4. Same as exp. 2, + NH_4Cl	>0.5

Exp. 1. The reaction mixture contained imidazole-HCl buffer (pH 8.3; 25 μmoles), Na α-ketoglutarate (7 μmoles), bovine liver glutamate dehydrogenase (1 μg), DPNH (0.14 μmole), and L-glutamine (E; 50 μmoles): final vol., 0.5 ml; 37°. The absorbance at 340 nm was followed. Exp. 2. A solution containing imidazole-HCl buffer (pH 7.2; 25 μmoles), $MgCl_2$ (10 μmoles), Na_2ATP (10 μmoles), 2-mercaptoethanol (12.5 μmoles), Na L-glutamate (0.075 μmole), L-glutamine (50 μmoles), Na α-ketoglutarate (7 μmoles), and glutamine synthetase (1.5 μg) was incubated at 37° for 30 min. The pH was adjusted to 8.3 by adding 0.05 ml of 1 M NaOH; DPNH (0.14 μmole) and bovine liver glutamate dehydrogenase (1 μg) were then added (final vol. 0.5 ml). Exp. 3. Same as exp. 2 except that ATP and $MgCl_2$ were omitted. Exp. 4. After incubation for 5 min. the reaction mixture used in exp. 2 (which showed no oxidation of DPNH), was mixed with 0.05 ml of 1 M NH_4Cl.

The present studies do not exclude the possibility that mammalian tissues contain glutamate synthase, an enzyme which has thus far been found in bacteria and plants, that catalyzes the glutamine-dependent reductive amination of α-ketoglutarate (12-15). The available data indicate that glutamate dehydrogenase and glutamate synthase are separate catalytic entities. However, purified glutamate synthase of A. aerogenes exhibits low but measurable activity when glutamine is replaced by ammonia (15). Glutamate synthase from E. coli (14) and from A. aerogenes (15) contain two non-identical subunits. That the light subunit of A. aerogenes glutamate synthase does not contain the glutamine binding site (as it does in carbamyl phosphate synthetase (27)) was shown by studies in which the glutamine-dependent activity was inhibited by L-2-amino-4-oxo-5-chloropentanoic acid (28), and in which [^{14}C] chloroketone bound only to the heavy subunit (which also contains flavin and iron-sulfide); glutamine protected against inhibi-

tion and binding (15). If the relatively high Km value for ammonia of liver glutamate dehydrogenase (> 20 mM) accurately reflects its in vivo affinity for ammonia, one might think that its major physiological function is glutamate deamination rather than glutamate synthesis. However, glutamate dehydrogenase might be closely linked in vivo with glutaminase (or glutamine transaminase and ω-amidase), or with a glutamine-binding subunit analogous to that of carbamyl phosphate synthetase (27); thus, the amide nitrogen of glutamine might be made directly available for synthesis of α-amino groups. The finding of preferential utilization of glutamine amide nitrogen for α-amino group synthesis in kidney preparations (29) suggests that there may be a pathway in mammals involving glutamate synthase or a catalytically equivalent glutamate dehydrogenase-glutamine utilizing enzyme or subunit linkage or complex.

Supported in part by grants from the Public Health Service, National Institutes of Health (AM 12034; AM 16739).

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