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1-Amino-1,3-dicarboxycyclohexane (Cycloglutamic Acid). A New Glutamic Acid Analog and a Substrate of Glutamine Synthetase*

Jerald D. Gass and Alton Meister

ABSTRACT: The ability of ovine brain glutamine synthetase to utilize α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate (but not other monomethyl-substituted glutamates) suggested that 1-amino-1,3-dicarboxycyclohexane (in which a chain of three methylene groups is introduced between the α- and γ-carbon atoms of glutamate) might be a substrate of the enzyme. 1-Amino-1,3-dicarboxycyclohexane (designated here by the trivial term cycloglutamic acid) was synthesized by hydrolysis of 3-carboxycyclohexane-1-spiro-5′-hydantoin (prepared by reacting 3-carboxycyclohexanone with ammonium carbonate and sodium cyanide).

The *cis* and *trans* racemic forms of cycloglutamic acid were obtained separately and tested for activity with the enzyme. Close to 50% of the racemic *cis* form was utilized by the enzyme in amide and hydroxamate synthesis, while the *trans* form was not active. The enzymatically synthesized amide was isolated and compared with the chemically synthesized product. The K_m and V_{max} values for *cis*-cycloglutamate in hydroxamate synthesis were similar to the corresponding values for glutamate. *cis*-Cycloglutamate (but not the *trans* isomer) can assume a diequatorial conformation equivalent to the extended conformation of glutamate. The fact that *cis*-cycloglutamate, whose 5-carbon chain is much more restricted in movement than that of glutamate, is a good substrate of glutamine synthetase affords strong support for the hypothesis that L-glutamate binds to the active site of the enzyme in the extended conformation.

The unusual substrate specificity of ovine brain glutamine synthetase is such that of the ten possible glutamate derivatives in which a methyl group is introduced into the glutamate carbon chain, only three are substrates: α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b; Meister, 1968) (Figure 1). These findings and the observations that both the L and D isomers of glutamate and α-aminoadipate are substrates (Wellner *et al.*, 1966), and that β-glutamate is enzymatically converted into D-β-glutamine (Khedouri and Meister, 1965), have led to an hypothesis concerning the conformation of these substrates on the enzyme. According to this hypothesis, (a) the carbon chain of L-glutamate is oriented on the enzyme in the fully (or almost fully) extended conformation in which the α-hydrogen atom of the substrate is directed away from the enzyme, (b) D-glutamate attaches to the enzyme in an extended conformation in which the α-hydrogen atom of this substrate is directed toward the enzyme.

The finding that the γ-*threo* and α-hydrogen atoms of L-glutamate can be replaced by methyl groups with retention of enzymatic susceptibility suggested the attractive possibility of constructing a cyclohexane ring by introducing a chain of three methylene groups connecting carbon atoms 2 and 4 of the glutamate carbon chain (Figure 2). The *cis*-L isomer of the resulting cyclohexane amino acid can exist in a form possessing a relatively rigid five-carbon chain identical with that of the extended conformation of L-glutamic acid; the position of the cyclohexane chain is entirely in the region in which methyl group substitutions that are consistent with retention of enzymatic susceptibility can be made. In this communication the synthesis of 1-amino-1,3-dicarboxycyclohexane[1] is described. Close to 50% of the racemic *cis* isomer is utilized by glutamine synthetase. The findings offer strong support for the hypothesis that L-glutamate attaches in the extended conformation to the active site of the enzyme.

Experimental Section

Methods and Materials. *m*-Methoxybenzoic acid was purchased from Distillation Products, Inc. Glutamine synthetase was prepared according to the method of Ronzio

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[1] This will be designated by the trivial term cycloglutamic acid.

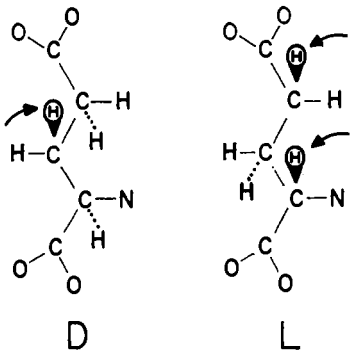

FIGURE 1: L- and D-glutamic acid; the hydrogen atoms that are encircled and indicated by arrows may be substituted by methyl groups without loss of enzymatic susceptibility (see the text).

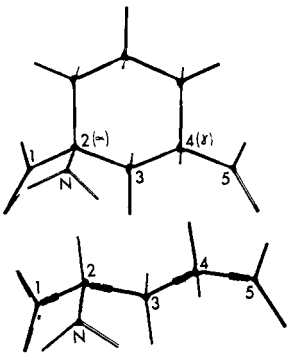

FIGURE 2: Dreiding models of L-glutamic acid in extended conformation (below) and of L-*cis*-1-amino-1,3-dicarboxycyclohexane (cycloglutamic acid) (above) (see the text). The numbering of the carbon atoms of cycloglutamic acid is intended only to facilitate comparison with glutamic acid.

et al. (1969) and assayed as previously described (Wellner and Meister, 1966). Phosphate was determined according to Fiske and Subbarow (1925). Pyruvate kinase was obtained from Boehringer Mannheim Corp. and phosphoenolpyruvate was obtained from Sigma Chemical Co. Kinetic data were analyzed by the computer programs described by Cleland (1967) as modified for use with an IBM 360/40 computer. Elemental analysis were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

3-Carboxycyclohexanone (*I*) (*Figure 3*). 3-Methoxybenzoic acid was purified by recrystallization from a mixture of diethyl ether and petroleum ether (mp 106–106.5°). Distilled ammonia (1 l.) was added to a solution of 3-methoxybenzoic acid (25 g) in tetrahydrofuran (100 ml) and absolute ethanol (75 ml). Sodium (18 g) was slowly added until the blue color persisted and then the mixture was stirred for 15 min. A solution of ammonium chloride (125 g) in water (300 ml) was slowly added. The excess ammonia was removed under reduced pressure and the solution was acidified with concentrated hydrochloric acid and then refluxed for 15 min. After cooling, the solution was exhaustively extracted with ether. The ether extracts were combined and dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure to give a colorless oil. On standing at 4° overnight, the oil solidified to a colorless solid, 10.8 g (48% of theory); mp 67–71°. The product was crystallized from a mixture of diethyl ether and petroleum ether, mp 71.5–72° (lit. mp 76° (Birch *et al.*, 1954); 73–75° (Dobson *et al.*, 1909; Titov and Kuznetsova, 1960)). The 2,4-dinitrophenylhydrazone had mp 200–201° (lit. mp 230° (Birch *et al.*, 1954); 202° (Banerjee *et al.*, 1957)). The semicarbazone had mp 175–176° (lit. mp 183–184° (Birch *et al.*, 1954); 182–183° (Titov and Kuznetsova, 1960)). The product exhibited only end absorbance in the ultraviolet, while partially reduced products exhibited a strong absorbance band at 225 mμ.

3-Carboxycyclohexane-1-spiro-5'-hydantoin (*II*). Small portions of a slurry of ammonium carbonate (31 g) in water (35 ml) were added to a solution of 3-carboxycyclohexanone (I) (10 g) in 95% ethanol (35 ml), until the evolution of carbon dioxide ceased. Then, sodium cyanide (4 g) was added together with the remaining slurry to the alcoholic solution. The mixture was maintained at 55–58° for 5 hr and then heated at 95° for 1 hr to decompose the remaining ammonium carbonate. This solution was acidified while hot by addition of concentrated hydrochloric acid. On cooling a crystalline product appeared, which was collected by filtration. The product was recrystallized from a large volume of water and dried over P_2O_5 in a vacuum desiccator overnight. A total of 14.3 g (97% of theory) was obtained. The compound did not melt or decompose below 310°. *Anal.* Calcd for $C_9H_{12}N_2O_4$: C, 50.9; H, 5.70; N, 13.2. Found: C, 50.7; H, 5.57; N, 13.4.

cis-1-Amino-1,3-dicarboxycyclohexane (*III*). The hydantoin (II) (13.0 g) was refluxed in 250 ml of 1 M sodium hydroxide for 24 hr. After cooling, the solution was slowly brought to pH 4.0 by addition of concentrated hydrochloric acid. A precipitate formed which was collected by filtration (5.4 g). This material was identical with the original hydantoin as indicated by its infrared spectrum and elemental analysis. The filtrate was further acidified to pH 3.2 and an additional precipitate formed which gave a positive purple color reaction with ninhydrin. This product was recrystallized from water and dried over P_2O_5 in a vacuum desiccator (5.6 g, 43%); mp 231–236° dec. Paper chromatography in a solvent consisting of 1-butanol–acetic acid–water (8:2:5, v/v) showed a single ninhydrin spot (R_F 0.52) as did chromatography in 80% phenol (R_F 0.35). *Anal.* Calcd for $C_8H_{13}NO_4 \cdot 0.5H_2O$: C, 49.0; H, 7.19; N, 7.13. Found: C, 49.5; H, 7.44; N, 7.06.

trans-1-Amino-1,3-dicarboxycyclohexane Hydrochloride. The hydantoin (1.6 g) was hydrolyzed as described by Munday (1961) by refluxing in 15 ml of 60% sulfuric acid for 24 hr. After dilution, sodium–barium hydroxide was added to the solution until the pH became basic. The pH was then adjusted to pH 7 by addition of sulfuric acid. The barium sulfate was removed by filtration and washed several times with hot water. The combined filtrates were concentrated under reduced pressure, and the pH was adjusted to 4 by adding hydrochloric acid. The precipitate which formed was removed by filtration. The volume was further reduced and an addi-

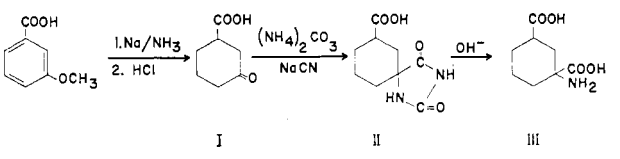

FIGURE 3: Synthesis of 1-amino-1,3-dicarboxycyclohexane.

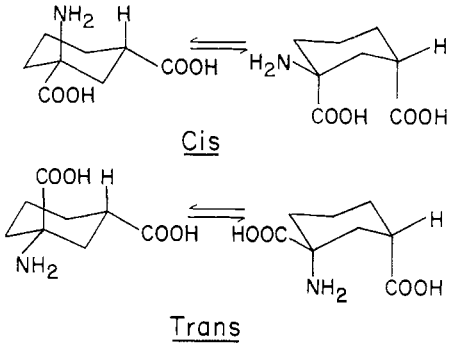

FIGURE 4: *cis* and *trans* forms of 1-amino-1,3-dicarboxycyclohexane.

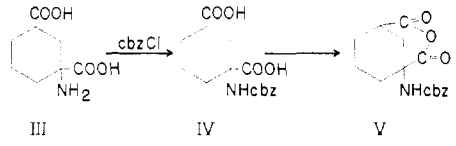

FIGURE 5: Conversion of *cis*-1-amino-1,3-dicarboxycyclohexane into the corresponding *N*-carbobenzoxyanhydride.

tional amount of material was collected. The solids were combined, dissolved in water, and acidified with HCl, and the amino acid hydrochloride was recrystallized from water and dried over P_2O_5 in a vacuum desiccator. This ninhydrin-positive material, which was different from that obtained by alkaline hydrolysis, was subsequently shown to be the *trans* isomer. Chromatography in butanol–acetic acid–water (8:2:5, v/v) gave a single ninhydrin spot (R_F 0.58) as did chromatography in 80% phenol (R_F 0.56). *Anal.* Calcd for $C_8H_{13}NO_4 \cdot HCl \cdot 1.5H_2O$: C, 38.3; H, 6.83; N, 5.59. Found: C, 39.0; H, 6.67; N, 5.50.

Chromatography of the *cis* and *trans* isomers was carried out on an automated amino acid analyzer (Spackman *et al.*, 1958); the *cis* and *trans* forms appeared as homogeneous peaks in the effluent at 161 and 155 min, respectively (glutamate, 79 min); thus, the isomers of cycloglutamate emerge between valine and methionine and are effectively separated from each other and from all of the common amino acids of proteins.[2]

Conversion of cis-1-Amino-1,3-dicarboxycyclohexane into the Corresponding Anhydride (V). The identification of the *cis* and *trans* forms of the cyclohexane amino acid (III) was based on the knowledge that only the *cis* form can exist in the diaxial conformation required for the formation of the intramolecular anhydride (Figure 4). Thus, the form of the amino acid which could undergo the transformations indicated in Figure 5 was identified as *cis*.

cis-1-(N-Carbobenzoxy)amino-1,3-dicarboxycyclohexane (IV). *cis*-1-Amino-1,3-dicarboxycyclohexane (III) (3 g) was added to a slurry of sodium bicarbonate (16 g) in water (10 ml). Carbobenzoxychloride (10 g) was added and the mixture was stirred at 24° for 24 hr. The mixture was extracted with ether and the aqueous phase was acidified with concentrated HCl and then extracted with several volumes of ethyl acetate. The combined ethyl acetate extract was dried over anhydrous $MgSO_4$, filtered, and then evaporated under reduced pressure to yield a colorless oil. On standing at 5°, crystals formed which were collected (3.4 g; 79%); mp 109–110°.

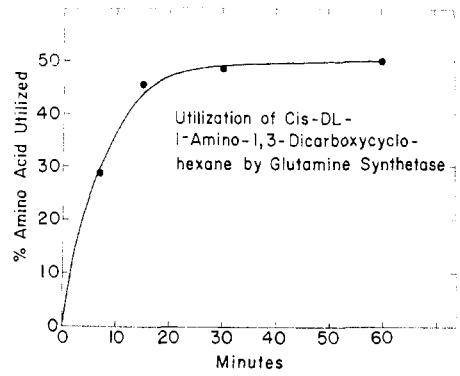

FIGURE 6: Utilization of *cis*-DL-cycloglutamate by glutamine synthetase. The reaction mixtures contained glutamine synthetase (56 units), ATP (11 mM), $MgCl_2$ (20 mM), *cis*-DL-cycloglutamate (1.35 mM), 2-mercaptoethanol (0.25 mM), hydroxylamine (100 mM), and imidazole·HCl buffer (pH 7.2, 50 mM); incubated at 37°.

cis-1-(N-Carbobenzoxy)amino-1,3-dicarboxycyclohexane Anhydride (V). The carbobenzoxy derivative (IV) (1.3 g) was dissolved in acetic anhydride (10 ml) and heated at 100° for 2 hr. The excess acetic anhydride was removed under reduced pressure and the remaining oil was dissolved in ethyl acetate. The ethyl acetate solution was extracted with saturated aqueous sodium bicarbonate, dried over anhydrous $MgSO_4$, and filtered. After removing the ethyl acetate under reduced pressure, the residual oil crystallized on standing at 5°. This was recrystallized from a mixture of diethyl ether and ethyl acetate resulting in 0.5 g (41%) of product, mp 108–109°. *Anal.* Calcd for $C_{16}H_{17}NO_5$: C, 63.4; H, 5.65; N, 4.62. Found: C, 63.4; H, 5.6; N, 4.90; mol wt: calcd, 303; found, 298.

Enzymatic Synthesis of cis-1-Amino-1-carboxy-3-carboxamidocyclohexane. When the DL-*trans* isomer of 1-amino-1,3-dicarboxycyclohexane was incubated with glutamine synthetase no reaction occurred, nor did the *trans* isomer inhibit the synthesis of L-glutamine. However, as seen in Figure 6 when the DL-*cis* isomer was incubated with glutamine synthetase, ATP, Mg^{2+}, and hydroxylamine the amino acid was rapidly utilized, and the reaction leveled off when close to 50% of the added substrate was converted into the hydroxamate.[3] The effect of cycloglutamate concentration on the rate of amide and hydroxamate synthesis is given in Figure 7.

The average V_{max} and K_m values calculated (for the L isomer) from a number of such experiments were, respectively, 102 ± 8 (std dev) and 5.48 ± 1.7 mM (hydroxamate synthesis) and 29.4 ± 4 and 5.13 ± 1.9 mM (amide synthesis). The comparative values for L-glutamate are 100 and 3.3 mM (hydroxamate synthesis) and 100 and 3.9 mM (amide synthesis) (Kagan and Meister, 1966a; Wellner *et al.*, 1966). The K_m values for hydroxylamine and ammonia (with cycloglutamate) were 3.72 ± 0.2 mM and 65.7 ± 6.3 mM, respectively. The

[2] We thank Mr. Lawrence Pinkus and Dr. Daniel Wellner for carrying out these procedures.

[3] The absorbance of the hydroxamic acid at 435 mμ was, within experimental error, the same as that of γ-glutamylhydroxamic acid.

K_m values for hydroxylamine and ammonia (with L-glutamate) are 0.15 and 0.18 mM, respectively (Wellner *et al.*, 1966).

In order to isolate the amino acid amide product of the reaction, a large-scale experiment was performed in which phosphoenolpyruvate and pyruvate kinase were added to regenerate ATP. The reaction mixture consisted of *cis*-1-amino-1,3-dicarboxycyclohexane (10 mmoles), $MgCl_2$ (2 mmoles), ammonium chloride (50 mmoles), ATP (1 mmole), mercaptoethanol (0.15 mmole), phosphoenolpyruvate (7.3 mmoles), pyruvate kinase (600 units), and glutamine synthetase (1050 units) in a final volume of 58 ml. The mixture was adjusted to pH 7.2 by adding potassium hydroxide and incubated at 37° with gentle shaking. After 72 hr the reaction had proceeded to about 60% of the theoretical as determined by inorganic phosphate determinations. The reaction was stopped by adding 100 ml of cold ethanol, and the precipitated protein was removed by centrifugation. The precipitate was washed with two portions of 10 ml of cold ethanol. The supernatant solutions were combined and the volume was reduced to 10 ml by evaporation under reduced pressure at 35°. This solution was placed on a Dowex 50 (H^+) column (2 × 10 cm) which was washed with water until the eluate returned to neutral pH and no chloride ion was detected with silver nitrate. The column was then washed with 2 M ammonium hydroxide. The eluate was evaporated under reduced pressure to yield a colorless solid. This was dissolved in the minimal amount of water and the pH was adjusted to 7.0 by adding HCl. The solution was then added to a Rexyn CG5 (H^+) column (3 × 63 cm), which was washed with water. The effluent was collected in 1.5-ml fractions. The two ninhydrin-positive peaks were identified by electrophoresis in 0.05 M sodium acetate (pH 5.5) for 40 min (60 V/cm). The dicarboxylic amino acid migrated 17 cm toward the positive pole and the amide moved 4 cm toward the negative pole.[4] Cycloglutamic acid appeared in fractions 240–425, and the amide was found in fractions 380–810. Fractions 426–810 contained only amide; these were combined and evaporated under reduced pressure. The amide was recrystallized from 95% ethanol (yield, 0.2 g). *Anal.* Calcd for $C_8H_{14}N_2O_3$: N, 15.1. Found, N, 15.0; $[\alpha]_D^{21.8}$ −15.3°. The amide melted with evolution of gas at 275–277°.

Nonenzymatic Synthesis of cis-1-Amino-1-carboxy-3-carboxamidocyclohexane. The amide, *cis*-1-amino-1-carboxy-3-carboxamidocyclohexane, was prepared by the aminolysis of the corresponding ethyl ester prepared by the method of Bergmann and Zervas (1933). A solution of 1-amino-1,3-dicarboxycyclohexane (4.49 g) in absolute ethanol (35 ml) was treated with gaseous hydrogen chloride (2.3 g) and then shaken for 30 min. The small amount of material which did not dissolve was removed by filtration and the filtrate was neutralized with triethylamine and placed at −5° for 18 hr. The crystals of triethylamine hydrochloride were removed by filtration and the filtrate was evaporated to dryness at reduced pressure. The remaining oil was dissolved in 28% aqueous ammonia (100 ml) and stirred at 24° for 18 hr. The solution was evaporated to dryness under reduced pressure and the remaining solid was recrystallized several times from water (1.0 g). *Anal.* Calcd: N, 15.1. Found: N, 15.4. An identical product was obtained by the modified procedure of Boissonnas *et al.* (1955). The product melted with evolution of gas at 280–282°.

[4] It was found that when sodium chloride was present the dicarboxylic acid and the corresponding monoamide exhibited identical mobility on paper electrophoresis at pH 5.5 under these conditions; appropriate precautions were therefore taken in subsequent work.

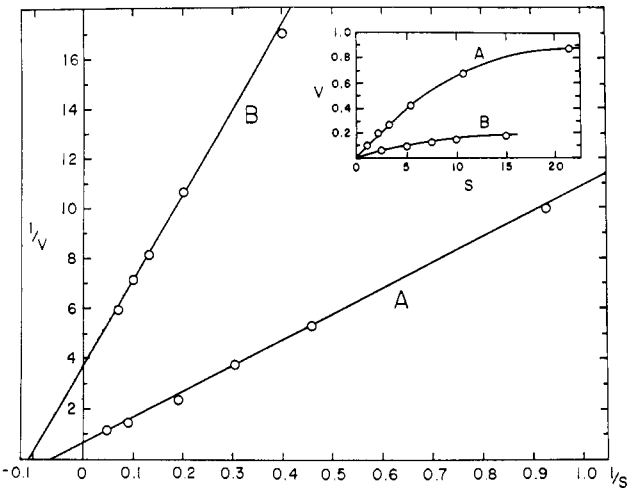

FIGURE 7: Effect of substrate concentration on the rate of hydroxamate formation from *cis*-DL-cycloglutamate. The reaction mixtures contained glutamine synthetase (1 unit), *cis*-cycloglutamate (as indicated), ATP (10 mM), $MgCl_2$ (20 mM), 2-mercaptoethanol (0.25 mM), ammonium chloride or hydroxylamine (100 mM), and imidazole·HCl buffer (pH 7.2; 50 mM); incubated at 37°. Double-reciprocal plot of data (A, NH_3; B, NH_2OH); inset: *V vs. S*.

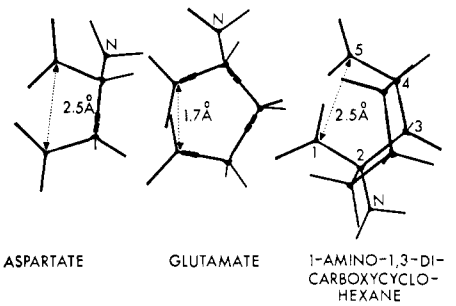

FIGURE 8: Dreiding models of L-aspartate, L-glutamate, and L-*cis*-1-amino-1,3-dicarboxycyclohexane in conformations processing minimal intercarboxyl carbon distances; see text.

Both the chemically synthesized and enzymatically synthesized amides were completely hydrolyzed by refluxing in 4 N HCl for 3 hr. When the amino acids thus formed were incubated with glutamine synthetase, ATP, Mg^{2+}, and hydroxylamine under the conditions described in Figure 6, the reaction stopped at 52.8% of completion with the amino acid derived from the chemically synthesized amide, and at 98.5% of completion with the amino acid obtained from the enzymatically synthesized amide.

The behavior of the two amides was identical on paper chromatography in 1-butanol–acetic acid–H_2O (8:2:5, v/v) (R_F 0.36) and in 80% phenol (R_F 0.72). On paper electrophoresis at pH 8.5 (0.05 M diethyl barbiturate buffer; 90 min; 40 V/cm) both products moved 13.1 cm. On electrophoresis at pH 3.0 (0.05 M phosphate buffer; 150 min; 40 V/cm),

both products moved 19.5 cm. Under these conditions glutamine and isoglutamine are readily separated (Coles *et al.*, 1962); thus, the mobilities of glutamine and isoglutamine were, respectively, 16.5 and 28.6 cm (pH 8.5), and 23.8 and 34.5 cm (pH 3.0). Both preparations of the amide of cycloglutamate as well as glutamine developed a blue color when boiled in aqueous solution with copper oxide, whereas a solution of isoglutamine treated in the same manner remained colorless. The ability to form copper complexes under these conditions is a characteristic of α-amino acids (Greenstein and Winitz, 1961).

Chromatography was also carried out on an amino acid analyzer (Spackman *et al.*, 1958);[2] both the enzymatically synthesized and the chemically synthesized amides emerged from the column in 113.5 min (*cf.* alanine, 107.5 min; cystine, 126.5 min).

Discussion

The present work has demonstrated that the glutamate analog *cis*-1-amino-1,3-dicarboxycyclohexane is an excellent substrate for glutamine synthetase, an observation which affords strong support for the hypothesis that the L-glutamate molecule attaches to the active site of the enzyme in an extended conformation. The present findings show that only one isomer of DL-*cis*-cycloglutamate is a substrate for glutamine synthetase. Since methyl substitutions of either the α- or γ-carbon atoms of D-glutamate prevent interaction with the enzyme, we tentatively conclude (in the absence of independent evidence at this time) that the susceptible isomer of *cis*-DL-cycloglutamate is the L isomer. It is evident that the five-carbon chain of *cis*-cycloglutamate is much more restricted in movement than that of glutamate; indeed, formation of a cyclic derivative analogous to pyrrolidonecarboxylate is not possible. Only the *cis* isomer of cycloglutamate can assume the diequatorial conformation equivalent to the extended conformation of glutamate. In contrast, the *trans* isomer must always assume an axial–equatorial conformation. The findings indicate that the *trans* isomer does not interact with the enzyme. Although the *cis* isomer can assume a diaxial conformation (as indicated by its conversion into the anhydride), the diequatorial conformation would be expected to be much more stable. Thus, the free energy difference between the axial and equatorial conformations of monocarboxycyclohexane is close to -2 kcal/mole (Eliel *et al.*, 1965); these authors note that in 1,3-disubstituted cyclohexanes the diaxial conformation is very unfavorable owing to crowding of the substituents. When the substituents are negatively charged (*i.e.*, as in *cis*-cycloglutamate at pH 7), electrostatic repulsion would also be expected to have an important effect.

In previous considerations of this subject, the failure of aspartate to interact with the enzyme was cited as support for the belief that glutamate attaches to the enzyme in the fully extended conformation. Thus, it was proposed that the carboxyl groups of glutamate are bound to sites on the enzyme which are further apart than the maximum intercarboxyl distance for aspartate (about 4 Å). Although other findings give additional support to this view, it would be theoretically possible for the carboxyl groups of glutamate to attach to enzyme sites that are sufficiently close to each other so as to prevent attachment of aspartate. Thus, the minimum intercarboxyl carbon distance for glutamate is about 1.7 Å, while that for aspartate is about 2.5 Å (Figure 8). However, the minimal intercarboxyl carbon distance for *cis*-cycloglutamate, achieved when this compound assumes the diaxial conformation, is close to 2.5 Å. This consideration, which seems to exclude the possibility that the carboxyl binding sites of glutamate are as close as 1.7 Å, supports the original view that the glutamate carboxyl binding sites are further apart than the maximum intercarboxyl carbon distance for aspartate, and therefore that the glutamate carbon chain is fully extended or nearly so.

It will be of interest to carry out further studies with cycloglutamate, and especially to examine the ability of this glutamic acid analog and its amide derivative to interact with other enzymes that utilize glutamate and glutamine.

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