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Utilization of a Cyclopentane Analog of Glutamate (*cis*-1-Amino-1,3-dicarboxycyclopentane) by Glutamine Synthetase†

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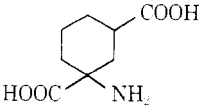
ABSTRACT: Previous studies, which showed that *cis*-1-amino-1,3-dicarboxycyclohexane is a substrate of ovine brain glutamine synthetase, have been extended to 1-amino-1,3-dicarboxycyclopentane, a glutamate analog in which two methylene groups have been introduced between the α- and γ-carbon atoms of glutamate. The cis and trans forms of 1-amino-1,3-dicarboxycyclopentane were separated and identified. Glutamine synthetase utilizes about 50% of the racemic cis form and does not interact with the trans form. Amide synthesis from *cis*-1-amino-1,3-dicarboxycyclopentane is more rapid than from *cis*-1-amino-1,3-dicarboxycyclohexane, and the K_m value for ammonia is much lower with the cyclopentane glutamate analog. These observations are in accord with computer-aided calculations of the conformation of the cyclopentane analog and its orientation on the active site of the enzyme. The findings are also consistent with earlier data on the mapping of the active site of glutamine synthetase which led to the conclusion that L-glutamate binds to the enzyme in the extended conformation.

The observation that *cis*-1-amino-1,3-dicarboxycyclohexane (A), a compound whose 5-carbon chain is much more restricted in movement than that of glutamate, is a good substrate of glutamine synthetase (Gass and Meister, 1970a) supports the hypothesis that L-glutamate binds to the active site of the enzyme in the fully (or almost fully) extended conformation with its α-hydrogen atom directed away from the enzyme (Meister, 1968; Gass and Meister, 1970b). The studies on 1-amino-1,3-dicarboxycyclohexane were initiated after it became apparent that glutamine synthetase can utilize α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate, but not the other monomethyl-substituted glutamates (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b); thus, 1-amino-1,3-dicarboxycyclohexane can be considered as a derivative of glutamate with a chain of three methylene groups introduced between the α- and γ-carbon atoms (Gass and Meister, 1970a).

In the present work we have prepared and studied an analogous glutamate derivative in which two methylene groups have been introduced between the α- and γ-carbon atoms of glutamate. The 5-carbon chain of this compound, 1-amino-1,3-dicarboxycyclopentane (B), is also very much more restricted in movement than that of glutamate. The findings reported here demonstrate that *cis*-1-amino-1,3-dicarboxycyclopentane is a good substrate of ovine brain glutamine synthetase and are in accord with earlier conclusions about the mapping of the active site of this enzyme (Meister, 1968; Gass and Meister, 1970b). The present studies suggest that this cyclopentane analog of glutamate may also be of interest in connection with other enzymes that act on glutamate.



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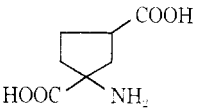

B

Experimental Section

Materials

Glutamine synthetase was isolated from sheep brain as described (Rowe *et al.*, 1970).

We are indebted to Dr. Leslie Hellerman for giving us a sample of 1-amino-1,3-dicarboxycyclopentane (mixture of cis and trans isomers) which had been prepared in his laboratory a number of years ago. We also obtained a sample of Dr. Hellerman's compound through the courtesy of Dr. Harry Wood of the Cancer Chemotherapy National Service Center (CCNSC); this compound was listed by CCNSC as *trans*-1-amino-1,3-dicarboxycyclopentane (NSC No. 27386), but chromatographic studies carried out in our laboratory showed that the sample contains about equal amounts of the cis and trans isomers, as stated by Dr. Hellerman (personal communication).

We thank Dr. J. W. Wilt for giving us a generous sample of 1-benzamido-2-norbornene (Wilt *et al.*, 1968).

Lactate dehydrogenase and pyruvate kinase were obtained

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from Boehringer Mannheim Corp. DPNH, ATP, and trisodium phosphoenolpyruvate were obtained from Sigma Chemical Co.

Methods

The enzymatic formation of hydroxamate from glutamate and 1-amino-1,3-dicarboxycyclopentane was determined using the glutamine synthetase hydroxamate assay previously described (Rowe *et al.*, 1970). In some experiments, the formation of ADP was determined as follows. After incubation, the assay mixture (1 ml) was treated with 0.1 ml of 2 N HCl to destroy the enzyme; the mixture was then adjusted to pH 7.6 by addition of 0.1 ml of 2 M Tris–KOH (pH 12.2). This buffered sample was mixed with 1.8 ml of a solution containing pyruvate kinase (1 unit/ml), lactate dehydrogenase (1 unit/ml), phosphoenolpyruvate (1.5 mM), DPNH (0.5 mM), KCl (50 mM), $MgCl_2$ (5 mM), and Tris-HCl buffer (0.3 M, pH 7.6); final volume, 3.0 ml. After incubation for 5 min at 37°, the change in absorbance at 340 nm was determined against controls in which either glutamine synthetase or ATP was omitted. Under these conditions, an absorbance difference of 0.062 is equivalent to 0.01 μmole of ADP/ml of final reaction mixture. The formation of ADP was linear and reproducible over the range 0.005–0.05 μmole of ADP, which corresponds to 0.015–0.15 unit of glutamine synthetase as determined by the γ-glutamyl hydroxamate assay method (Rowe *et al.*, 1970). We observed close agreement between the hydroxamate and ADP assay methods indicating that the molar extinction coefficient of the hydroxamate of *cis*-1-amino-1,3-dicarboxycyclopentane is, within experimental error, the same as that of γ-glutamyl hydroxamate.

Optical rotation was determined with a Cary Model 60 spectropolarimeter.

Synthesis of the Cis and Trans Forms of 1-Amino-1,3-dicarboxycyclopentane. 1-Amino-1,3-dicarboxycyclopentane was prepared previously by the Bucherer reaction on cyclopentanone-3-carboxylic acid (Connors and Ross, 1960); it seems probable that this product was a mixture of the cis and trans forms. We describe here an improved synthesis of this compound and the separation and identification of the cis and trans diastereoisomeric racemic forms. The synthetic pathway employed here is illustrated in Figure 1. Commercially available 4-vinylcyclohexene (I) was oxidized to 1,2,4-butanetricarboxylic acid (II), which was esterified (III) and then subjected to a Dieckmann cyclization to give a mixture of 2,3- and 2,4-cyclopentanonedicarboxylates (IV + V). Compounds IV + V were hydrolytically decarboxylated to yield the key intermediate, cyclopentanone-3-carboxylic acid (VI), which was converted by the Bucherer synthesis to the corresponding hydantoin (VII). Alkaline hydrolysis of VII gave a mixture of *cis*- and *trans*-1-amino-1,3-dicarboxycyclopentane; the cis and trans forms were separated by fractional crystallization. The cis isomer (IX) gave the corresponding *N*-acetyl cyclic anhydride, while the trans isomer (VIII) gave the acyclic anhydride. Additional support for this stereochemical assignment was derived from the conversion by oxidative cleavage of 1-benzamido-2-norbornene (X) to the cis isomer of 1-amino-1,3-dicarboxycyclopentane (IX).

1,2,4-Butanetricarboxylic Acid (II). 4-Vinylcyclohexene (Aldrich Chemical Co) was oxidized as follows (Conly, 1953). A solution of 2.0 g of ammonium metavanadate in 1.3 l. of concentrated nitric acid was warmed to 50°. The heat source was removed and 108 g of 4-vinylcyclohexene was added dropwise with stirring; the addition was made at a rate sufficient to maintain the temperature of the reaction mixture at 50–60°. The mixture was then allowed to cool and the solution, except for about 75 ml, was poured off and saved. This residue was heated to 100° and the remainder was added back dropwise, maintaining the temperature at 100°. After the addition was completed the mixture was heated for an additional hour at 100°, then cooled to 50°, and finally 300 ml of 48% formic acid was added cautiously to decompose the excess nitric acid. The reaction mixture was then concentrated in a flash evaporator. A green-grey solid formed which was decolorized with Norit in boiling acetone. A yellow solid was obtained, which was filtered and dried; mp 110–115° (lit. mp 118–120°). This material was used in the next step.

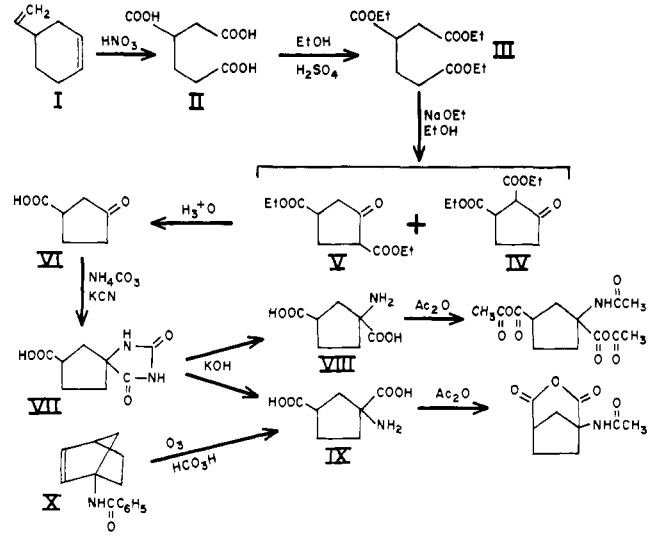


FIGURE 1: Synthesis of cis and trans forms of 1-amino-1,3-dicarboxycyclopentane.

Triethyl 1,2,4-Butanetricarboxylate (III). Compound II (150 g) was dissolved in a mixture of 1 l. of absolute ethanol and 10 ml of concentrated sulfuric acid, and the mixture was refluxed for 18 hr. The excess ethanol was distilled off and the residue was taken up in ether (200 ml). The ether was washed with saturated sodium bicarbonate until carbon dioxide formation ceased. The combined washings were extracted once with ether, and the ether was added to the main fraction. The combined ethereal solution was dried over anhydrous magnesium sulfate, filtered, and evaporated to yield an oil. The product was distilled under vacuum: bp 126–131° (1.25–1.50 mm) (lit. (Shemyakin *et al.*, 1957) bp 116–117° (1 mm)); yield, 90 g (45%).

Diethyl 2,3- and 2,4-Cyclopentanonedicarboxylates (IV and V). Sodium (8.5 g) under 100 ml of xylene was heated until molten and then vigorously stirred with a mechanical stirrer as the heat was removed and the solution was cooled to room temperature. The xylene was decanted from the fine beads of sodium and replaced by 220 ml of dry benzene. The triester, III (90 g), was added dropwise with mechanical stirring to the mixture at room temperature over a period of 1 hr, and the mixture was then refluxed with efficient stirring for 2 hr. The reaction was allowed to cool, and then acidified by addition of 4 N hydrochloric acid. The benzene and aqueous layers were separated and the aqueous layer was extracted with benzene. The combined benzene solutions were dried over anhydrous magnesium sulfate, filtered, and evaporated to an oil. The product was distilled under vacuum: bp 110–111°

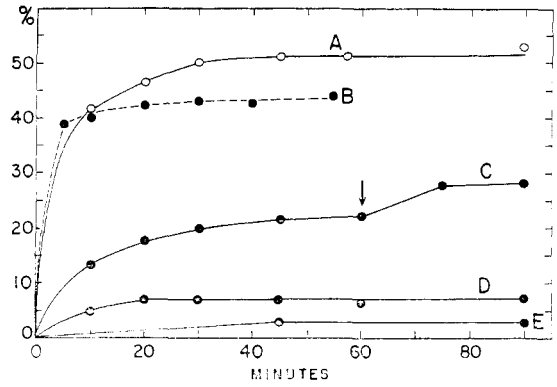


FIGURE 2: Utilization of 1-amino-1,3-dicarboxycyclopentane by glutamine synthetase. The reaction mixtures (final volume, 11 ml; in B, 5.5 ml) contained glutamine synthetase (as indicated below), ATP (10 mM), $MgCl_2$ (20 mM), 2-mercaptoethanol (25 mM), hydroxylamine (100 mM), 1-amino-1,3-dicarboxycyclopentane (as indicated below) and imidazole·HCl buffer (pH 7.2, 50 mM); incubated at 37°. (A) DL-*cis*-1-Amino-1,3-dicarboxycyclopentane (IX; 1.5 mM), enzyme (75 units); (B) DL-*cis*-1-amino-1,3-dicarboxycyclopentane (from ozonolysis of 1-benzamido-2-norbornene (X); 1.2 mM), enzyme (54 units); (C) DL-1-amino-1,3-dicarboxycyclopentane (mixture of cis and trans (CCNSC No. 27386); 1.5 mM) enzyme (150 units; 75 additional units were added at 60 min as indicated by arrow); (D) DL-*trans*-1-amino-1,3-dicarboxycyclopentane (VIII; 1.5 mM), enzyme (75 units); (E) DL-*trans*-1-amino-1,3-dicarboxycyclopentane (recrystallized, 3.0 mM), enzyme (75 units). The formation of hydroxamic acid was determined as described in the text.

(0.2 mm) (lit. (Shemyakin *et al.*, 1957) bp 110–115° (0.3 mm)); yield, 59.1 g (80%).

Cyclopentanone-3-carboxylic Acid (VI). The mixture, IV plus V (59.1 g), was refluxed for 3 hr in 350 ml of 8% sulfuric acid. After cooling the solution was saturated with sodium chloride and extracted four times with 100-ml portions of ether. The combined ethereal extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to a colorless oil (21.5 g), which gave a crystalline semicarbazone: mp 190–194° (lit. (Kay and Perkins, 1906) mp 195°).

3-Carboxycyclopentane-1-spiro-5'-hydantoin (VII). The keto acid, VI (6.4 g), was dissolved in 40 ml of 50% ethanol, and 17.1 g of ammonium carbonate monohydrate was added slowly with stirring to control foaming. Then 6.5 g of potassium cyanide was added and the mixture was stirred mechanically and heated to 55–60° for 6 hr. The mixture was acidified with concentrated hydrochloric acid and concentrated in a flash evaporator until a precipitate formed. This was collected, dried, and recrystallized from water: mp 220–230° dec (lit. (Connors and Ross, 1960) mp 228–230°); yield, 7.8 g (80%).

DL-*trans-1-Amino-1,3-dicarboxycyclopentane (VIII).* The hydantoin, VII (3.4 g), was refluxed with 100 ml of 1 M potassium hydroxide for 17 hr. The reaction mixture was cooled in an ice bath and brought to pH 7 by cautious addition of concentrated hydrochloric acid. A gelatinous precipitate formed, which was filtered over Celite; the filtrate was further acidified to pH 3.4. The precipitate did not give a positive ninhydrin test, nor did it melt or burn when held in a Bunsen flame, and it was therefore considered to be inorganic. The acidified filtrate was concentrated to about one-half of its volume; a white crystalline material then formed which was recrystallized from water: mp 246–250° (lit. (Connors and Ross, 1960) mp 264°); yield, 1.5 g (50%). This material gives a single ninhydrin-positive spot on ascending paper chromatography in 80% phenol (R_F 0.43). Chromatography of this compound on an automated amino acid analyzer (Spackman *et al.*, 1958) gave a single symmetrical peak appearing at 100 min; glutamate appeared at 66.5 min. *Anal.* Calcd for $C_7H_{11}NO_4 \cdot {}^7/_8H_2O$: C, 44.49; H, 6.81; N, 7.41. Found: C, 44.65; H, 6.71; N, 7.42.

Reaction of trans-1-Amino-1,3-dicarboxycyclopentane (VIII) with Acetic Anhydride. The amino acid, VIII (400 mg), was refluxed with 10 ml of acetic anhydride for 4 hr. The excess acetic anhydride was removed under vacuum, and the residue was dissolved in ethyl acetate and washed with 10% sodium bicarbonate. The ethyl acetate layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to yield 200 mg of an oil which could not be crystallized; ir spectra ($CHCl_3$): 1813 and 1750 cm^{-1}, acyclic anhydride carbonyl; 1680 cm^{-1}, amide carbonyl; 910 cm^{-1}, C—O—C acyclic anhydride.

DL-*cis-1-Amino-1,3-dicarboxycyclopentane (IX).* The mother liquors of all the trans isomer recrystallizations were combined and applied to a column of Dowex 50 (H^+), which was washed with water until chloride ion could no longer be detected in the effluent. The amino acid was eluted with 1 M ammonium hydroxide. The eluted fractions were combined and concentrated to dryness; the residue was dissolved in about 10 ml of water and the pH of this solution was brought to 3.4 by addition of concentrated hydrochloric acid. The product crystallized as fine white needles. Recrystallization from water gave 679 mg (23%) of material melting at 275–280° with decomposition. This material gave a single ninhydrin-positive spot on ascending paper chromatography in 80% phenol: R_F 0.21. Chromatography of this compound on the automated amino acid analyzer gave a single symmetrical peak which appeared at 114 min; glutamate appeared at 66.5 min. *Anal.* Calcd for $C_7H_{11}O_4N \cdot 0.75H_2O$: C, 45.03, H, 6.74; N, 7.50. Found: C, 44.90; H, 6.67; N, 7.33.

Reaction of cis-1-Amino-1,3-dicarboxycyclopentane (IX) with Acetic Anhydride. The amino acid, IX (400 mg), was refluxed with 10 ml of acetic anhydride for 4 hr. The excess acetic anhydride was removed under vacuum, and the residue was taken up in 25 ml of ethyl acetate and washed with 10% sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to yield 170 mg of an oil which could not be crystallized; ir spectra ($CHCl_3$): 1780 and 1718 cm^{-1}, cyclic anhydride carbonyl; 1690 cm^{-1}, amide carbonyl.

Preparation of the Cis Amino Acid (IX) from 1-Benzamidonorbornene-2 (X). 1-Benzamido-2-norbornene (Wilt *et al.*, 1968) (200 mg) was suspended in methanol (2.5 ml) and cooled in a Dry Ice–acetone bath. The mixture was treated with ozone for about 25 min; a Welsbach Model T-23 ozonator was used with a flow rate of 0.04 ft^3 min at 115 V and 8 psi of oxygen. When all the solid material dissolved and the solution turned blue, the flow of ozone was stopped and the solvent was removed in a flash evaporator. The residual oil was dissolved in 1.7 ml of 90% formic acid and 1.0 g of 50% hydrogen peroxide was added. The solution was heated gently until a vigorous reaction commenced and subsided; it was then refluxed for 30 min. The mixture was concentrated in a flash evaporator and the residue was dissolved in water. Ethanol was added until the solution became turbid; on cooling, fine white needles formed, which were collected and dried: mp 118–120° (with sublimation); yield, 70 mg (52%). This material, which did not give a ninhydrin test, gave an ir absorption curve that was identical with that of an authentic sample of benzoic acid. The mother liquors, which gave a positive ninhydrin test, were applied to a column of Dowex 50 (H^+), which was washed with water until the effluent was

TABLE I: Utilization of Cyclopentane and Cyclohexane Analogs of Glutamate by Glutamine Synthetase.[a]

Substrate Varied	Hydroxamate Synthesis		Amide Synthesis	
	Rel V_{max}	K_m (mM)	Rel V_{max}	K_m (mM)
L-Glutamate	(100)	3.3	100	3.9
L-*cis*-1-Amino-1,3-dicarboxycyclohexane (cHex)	102	5.5	29	1.9
L-*cis*-1-Amino-1,3-dicarboxycyclopentane (cPe)	70	10	43	7.7
NH_4Cl (with L-glutamate)			100	0.3
NH_4Cl (with c-Hex)			29	66
NH_4Cl (with c-Pe)			43	6.9
NH_2OH (with glutamate)	(100)	0.4		
NH_2OH (with c-Hex)	102	3.7		
NH_2OH (with c-Pe)	70	0.5		

[a] The reaction mixtures (final volume, 1.0 ml) contained enzyme (0.5–1.0 unit), 2-mercaptoethanol (25 mM), ATP (10 mM), $MgCl_2$ (20 mM), imidazole-HCl buffer (pH 7.2, 50 mM), glutamate or analog (when constant, 50 mM of L isomer), NH_4Cl and NH_2OH (when constant, 100 mM); incubated for 15 min at 37°. ADP was determined as described under methods. The data obtained with 1-amino-1,3-dicarboxycyclohexane were taken from Gass and Meister (1970a).

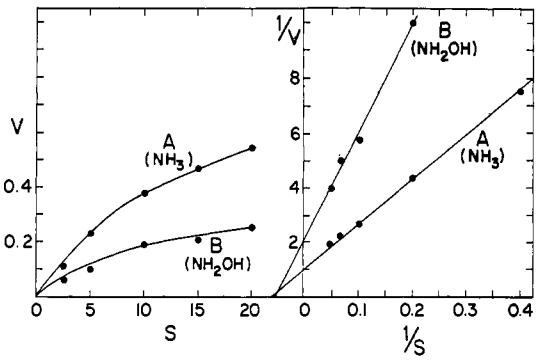


FIGURE 3: Effect of DL-*cis*-1-amino-1,3-dicarboxycyclopentane concentration on the rate of ADP formation by glutamine synthetase. The reaction mixture contained glutamine synthetase (0.5 unit), DL-*cis*-1-amino-1,3-dicarboxycyclopentane (as indicated), ATP (10 mM), $MgCl_2$ (20 mM), 2-mercaptoethanol (25 mM), ammonium chloride (A) or hydroxylamine (B) (100 mM), and imidazole·HCl buffer (pH 7.2, 50 mM) in a volume of 0.5 ml; incubated at 37°. Left side: plot of initial velocity, V, *vs.* concentration, (S); right side: double reciprocal plot ($1/V$ *vs.* $1/(S)$. ADP was determined as described in the text

neutral. The amino acid was eluted with 1 M ammonium hydroxide. The fractions containing the amino acid were pooled and evaporated to a light brown oil. On paper chromatography in 80% phenol this product gave a single ninhydrin-positive spot with the same R_F value (0.21) as that of IX obtained as described above; chromatography on the automated amino acid analyzer gave a single symmetrical peak which appeared in the position of IX obtained as described above at 114 min (glutamate, 66.5 min). As indicated below, this product and IX obtained by pathway I → IX (Figure 1) exhibited very similar enzymatic susceptibility.

Utilization of 1-Amino-1,3-dicarboxycyclopentane by Glutamine Synthetase. As indicated in Figure 2, about 28% of a preparation consisting of a mixture of *cis*- and *trans*-1-amino-1,3-dicarboxycyclopentane (mixture of four isomers) was utilized by glutamine synthetase in the presence of hydroxylamine and a large excess of enzyme (curve C). On the other hand, about 50% of the racemic cis isomer synthesized by pathway I → IX (Figure 1) was utilized (curve A, Figure 2). About 44% of the racemic cis isomer prepared by ozonolysis of X (pathway X → IX; Figure 1) was utilized (curve B, Figure 2). Only about 7% of the racemic trans form (curve D) was used, and after this compound was recrystallized, less than 3% was utilized (curve E). These observations therefore indicate that the trans form is not a substrate and that only one optical isomer of DL-*cis*-1-amino-1,3-dicarboxycyclopentane is a substrate.[1] In separate experiments it was found that the DL-trans isomer (10 mM) did not inhibit the synthesis of L-γ-glutamyl hydroxamate in the standard glutamine synthetase assay system.

The effect of varying the concentration of DL-*cis*-1-amino-1,3-dicarboxycyclopentane concentration on the rate of amide and hydroxamate synthesis is described in Figure 3. The apparent K_m values calculated from these data for the L isomer were 7.7 (amide synthesis) and 10 mM (hydroxamate synthesis). The V_{max} values (relative to those for L-glutamate) are given in Table I, which also gives the comparative values for *cis*-1-amino-1,3-dicarboxycyclohexane, and the K_m values for ammonia and hydroxylamine with glutamate and the two cyclic glutamate analogs.

Resolution of DL-cis-1-Amino-1,3-dicarboxycyclopentane with Glutamine Synthetase. The racemic cis form was incubated on a relatively large scale in the glutamine synthetase system. To facilitate the necessary separations, a low concentration of ATP was used and phosphoenolpyruvate and pyruvate kinase were added as an ATP-regenerating system. The reaction mixture consisted of DL-*cis*-1-amino-1,3-dicarboxycyclopentane (0.5 mmole), NH_4Cl (2.5 mmoles), $MgCl_2$ (0.1 mmole), ATP (0.09 mmole), phosphoenolpyruvate (0.36 mmole), pyruvate kinase (30 units), 2-mercaptoethanol (0.15 mmole), and glutamine synthetase (50 units), in a final volume of 5.6 ml. The solution was adjusted to pH 7.2 by addition of KOH, and it was then incubated at 37°. The course of the reaction was followed by determinations of inorganic phosphate (Fiske and Subbarow, 1925). The reaction proceeded to almost 50% of amino acid utilization after 5-hr incubation; incubation was continued for an additional 26 hr. The reaction mixture was then treated with one volume of absolute ethanol at 0° and then centrifuged to remove the precipitated protein. The protein sediment was washed twice with cold 50% ethanol and the supernatant solution and washings were combined and evaporated to 3 ml in a flash evaporator at 30°. The concentrated solution was added to the top of a column (1.0 × 19 cm) of Dowex 50 (H^+), which was washed with water until the effluent became free of chloride ion. The amino acids were eluted from the column with 40 ml of 1 N NH_4OH. The eluate was evaporated to dryness in a flash evaporator at 30° and the residue obtained was dis-

These findings are analogous to those made on 1-amino-1,3-dicarboxycyclohexane and are in accord with the stereochemical considerations previously discussed (Gass and Meister, 1970b).

solved in 4 ml of 30% ethanol. This solution was treated with 8 ml of acetone, and after standing for 2 days at 4°, an oil was collected by centrifugation. Paper chromatographic study of this material using a solvent consisting of 80% phenol indicated the presence of two amino acids; one of these corresponded to *cis*-1-amino-1,3-dicarboxycyclopentane (R_F 0.21), and the other (the corresponding amide) exhibited an R_F value of 0.56. In a solvent consisting of *tert*-butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15) the respective R_F values were 0.69 and 0.33 (the R_F values for the trans isomer, glutamate, and glutamine were, respectively, 0.68, 0.51, and 0.27). On paper electrophoresis (0.05 M ammonium acetate (pH 5.5); 1 hr; 88 V/cm), the cis and trans forms moved −12 cm toward the anode, while the amide product moved 1 cm in the opposite direction.

The oil (mixture of the unutilized dicarboxylic acid and the amide) was dissolved in 1 ml of water and the solution was adjusted to pH 7 by addition of 0.1 M NaOH. This solution was added to the top of a column (1 × 10 cm) of Dowex 1 (acetate). The amide was eluted from the column with water and the fractions containing it were combined and evaporated to dryness in a flash evaporator. The dry residue was dissolved in 2 ml of water and 4 ml of ethanol was added. The product, which crystallized after 48 hr at 4°, was filtered off and recrystallized from ethanol–water: mp 212–215°. The yield was 38.2 mg (79%); the product gave a single spot (R_F 0.56) on paper chromatography in 80% phenol. The Dowex 1 (acetate) column was then eluted with 50 ml of 1 M acetic acid, and the fractions containing the dicarboxylic amino acid were evaporated to dryness in a flash evaporator. The residue was crystallized twice from ethanol–water; yield, 41.6 mg (83%); the material was homogeneous on paper chromatography in 80% phenol (R_F 0.21). Its mobility on paper electrophoresis at pH 5.5 and its R_F value on paper chromatography in *tert*-butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15) were identical with that of the cis amino acid prepared as described above: mp 255–258° dec. *Anal.* Calcd for $C_7H_{11}NO_4$: N, 8.09. Found: 8.19.

The enzymatically synthesized amide was hydrolyzed to the corresponding dicarboxylic amino acid by refluxing in 6 N hydrochloric acid for 1 hr. The hydrolysate was evaporated to dryness and the residue was dissolved in water, adjusted to pH 7, and purified by chromatography on a column of Dowex 1 (acetate) as described above. A crystalline product was obtained, which moved on chromatography and electrophoresis as a homogeneous compound with the cis amino acid prepared by organic synthesis: mp 254–256° dec. Calcd for $C_7H_{11}NO_4$: N, 8.09. Found: 8.14. When this amino acid was incubated in the glutamine synthetase system (Figure 2) more than 80% was utilized after 60-min incubation. The enzymatically susceptible isomer exhibited a molar rotation ($[M]_{25°}^{226nm}$) of +425 ± 30° in 0.5 N HCl; the rotation exhibited by the unutilized isomer under these conditions was −364 ± 30°.

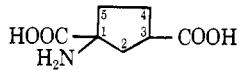
Discussion

The identification of IX as the cis isomer of 1-amino-1,3-dicarboxycyclopentane is supported by its conversion on treatment with acetic anhydride to the *N*-acetyl cyclic anhydride. The cis form, in contrast to the trans form, can yield a cyclic anhydride. The *N*-acetyl cyclic anhydride of IX was identified by its infrared spectrum in chloroform. Typical cyclic anhydride absorption (Tiwari and Sharma, 1970), *i.e.*, the intense band at 1780 cm^{-1} and the more intense band at 1718 cm^{-1}, was observed; the amide carbonyl band appeared at 1690 cm^{-1}. Treatment of VIII with acetic anhydride gave a derivative whose ir spectrum in chloroform exhibited bands at 1813 and 1750 cm^{-1}, the former being more intense, which are characteristic of an acyclic anhydride (Tiwari and Sharma, 1970; Nakanishi, 1962). The band observed at 910 cm^{-1} has also been attributed to acyclic (C—O—C) anhydride absorption. Further proof of the stereochemical assignments was obtained here by oxidative cleavage of the double bond of 1-benzamido-2-norbornene (X); ozonolysis followed by oxidation with performic acid gave an isomer identical with IX.

The enzyme studies reported here establish that one antipode, presumably the L isomer, of *cis*-1-amino-1,3-dicarboxycyclopentane is a substrate of glutamine synthetase. The racemic trans form is not a substrate, nor does the trans form inhibit the enzyme. The susceptibility of *cis*-1-amino-1,3-dicarboxycyclopentane to glutamine synthetase is in accord with previous work in this laboratory on the action of glutamine synthetase on 1-amino-1,3-dicarboxycyclohexane and other glutamate derivatives (Gass and Meister, 1970a,b; Meister, 1968). Although the conformation of *cis*-1-amino-1,3-dicarboxycyclopentane is not known, its conformation must be such as to be able to bind to the active site defined by the earlier studies since the data indicate that it is a substrate. It is well known that the cyclopentane ring is not planar, but that it is invariably puckered. The nature and extent of such puckering are substantially influenced by the substituents. Thus, Allinger *et al.* (1968) have suggested that the most stable conformation of *cis*-1,3-dimethylcyclopentane is probably the half-chair (C_2) conformation. On the other hand, Haresnape (1953) has proposed that the most stable conformation of this compound is the envelope form in which four of the ring atoms are coplanar and the fifth carbon atom is out of the plane. In both the half-chair and envelope conformations, the methyl groups are in quasiequatorial positions. It seems probable that the conformation of the cyclopentane ring of *cis*-1-amino-1,3-dicarboxycyclopentane is puckered by the cis 1,3-disubstitution in order to relieve what would be expected to be very strong repulsive interactions between the carboxyl groups.

An attempt was made to fit with the aid of a computer *cis*-L-1-amino-1,3-dicarboxycyclopentane into the active site previously calculated (Gass and Meister, 1970b). In so doing we have made the arbitrary assumption that *cis*-1-amino-1,3-dicarboxycyclopentane exists in the envelope conformation. There would seem at present to be no additional advantage in carrying out an analysis based on a half-chair conformation, which would require a very large amount of computation. At this time there appears to be no compelling evidence in support of either of the suggested conformations. The present treatment, which is based on the relative positions of the carboxyl and amino group binding sites, seems to be a reasonable approach, and the results obtained may be consistent with more than one conformation. The findings described below indicate that there is at least one conformation consistent with the active site previously designed. This binding site consists of five points derived from the positions of the atoms of the natural substrate L-glutamate, *i.e.*, the α-carboxyl carbon atom, the α-amino nitrogen atom, the γ-carboxyl oxygen atom which is phosphorylated (OP site), the carboxyl oxygen atom (OB site), and the nitrogen atom of the tetrahedral intermediate formed by reaction of L-γ-glutamyl phosphate with ammonia (the ammonia binding site). The coordinates of several conformers of *cis*-1-amino-

1,3-dicarboxycyclopentane were calculated by assuming atoms C_1–C_3–C_4–C_5 to be coplanar and by varying the angle



between this plane and the plane containing C_1–C_2–C_3. When this angle is 0°, *i.e.*, a planar form of the ring, all C–C–C angles in the ring are that of a regular pentagon, *i.e.*, 108°. As the angle between the planes changes the angles C_2–C_1–C_5 and C_2–C_3–C_4 must also change. Distances and angles for some of these conformations are shown in Table IIA.[2] It is evident that in the first three conformations the intercarboxyl distances are considerably less than that found in the extended form of L-glutamate, *i.e.*, 5.03 Å. Since these conformers will fit less well than those having planar angles of 30–50°, a fit to the enzyme binding sites was attempted only with the last three conformers.

The cyclopentane analog was placed into this site in two ways. (a) The C_1 carboxyl carbon atom was placed at the α-carboxyl binding site and the C_1 nitrogen atom was placed at the α-amino binding site. The entire molecule was then rotated about an axis formed by these two points while the C_3 carboxyl group was simultaneously rotated until the position was reached in which the sum of the distances between the two oxygen atoms of the carboxyl group and the two enzyme oxygen binding sites was a minimum. The distances obtained are given in Table IIB. (b) The C_1 carboxyl carbon atom was placed at the α-carboxyl binding site and the C_3 carboxyl carbon atom was initially placed as near to the position of the γ-carboxyl carbon atom of enzyme-bound L-glutamate as possible. The molecule was then similarly rotated to the position of minimum distance around the axis formed by the α-carboxyl-α-amino binding sites, rather than about the axis formed by the α-amino group as in point a. In this case, the C_1 nitrogen atom of the cyclopentane analog was not precisely at the α-amino binding site. The distances obtained by this procedure are given in Table IIC. Table IID,E gives the results of a similar minimization procedure carried out with the tetrahedral intermediate derived from the cyclopentane analog.

As can be seen from Table IIB, the distances to the oxygen binding sites are quite large when the nitrogen atom is placed at the α-amino binding site. However, by offsetting this nitrogen atom slightly, the distances are greatly decreased (Table IIC). In this conformation all three of the conformers chosen would seem to bind reasonably well. The 50° conformer can bind very well with only a distance of 0.976 Å from the oxygen atom to the OP site; this is the critical distance for reaction with the terminal phosphate group of ATP (Gass and Meister, 1970b). It is obviously impossible for the oxygen atoms of the tetrahedral intermediate to fit exactly into the oxygen binding sites, which were derived from the positions of the oxygen atoms of L-glutamate. The calculated fit of the tetrahedral intermediate derived from L-glutamate was found to be 0.42 Å from the OP site and 0.25 Å from the OB site (Gass and Meister, 1970). From Table IID,E it can be seen that the 40 and 50° conformers fit into the enzyme sites better than does the tetrahedral intermediate derived from L-glutamate!

TABLE II: Distances and Angles of Conformers of *cis*-1-Amino-1,3-dicarboxycyclopentane (see text).

Planar Angle (deg)	Angles C_2–C_1–C_5 and C_2–C_3–C_4 (deg)	Inter-Carboxyl Distance (Å)
A. 0	108.0	4.18
10	107.5	4.43
20	106.0	4.65
30	103.5	4.82
40	100.3	4.96
50	96.3	5.07

	$-N^a$	OP^b	OB^c	
B. 30	0.0	1.6	1.5	
40	0.0	1.0	1.0	
50	0.0	0.49	0.64	
C. 30	0.56	0.20	0.46	
40	0.36	0.078	0.29	
50	0.19	0.076	0.24	
	$-N^a$	OP^b	OB^c	$N\text{-}C_4{}^d$
D. 30	0.0	1.3	1.1	2.6
40	0.0	0.76	0.68	2.6
50	0.0	0.29	0.31	2.6
E. 30	0.56	0.34	0.49	2.6
40	0.36	0.30	0.28	2.6
50	0.19	0.31	0.13	2.6

[a] Distance (ångstroms) between C_1-nitrogen atom and α-amino binding site. [b] Distance (ångstroms) between the carboxyl oxygen atom and OP site. [c] Distance (ångstroms) between the carboxyl oxygen atom and OB site. [d] Distance (ångstroms) from nitrogen atom of tetrahedral intermediate and C_4-ring carbon atom.

It was previously noted that substrates that are substituted on the carbon atoms corresponding to the γ-carbon atom of L-glutamate exhibit lower rates of amide synthesis, and it was postulated that this should result in crowding in the tetrahedral intermediate and steric hindrance to the approach of the ammonia nitrogen atom. Thus, the distance between the nitrogen atom of the tetrahedral intermediate and the γ-methyl group of *threo*-γ-methyl-L-glutamate or the C_4 methylene of *cis*-1-amino-1,3-dicarboxycyclohexane is 2.2 Å, while the sum of the van der Waals radii of the nitrogen atom (1.5 Å) and a methyl or methylene group (2.0 Å) is 3.5 Å. In the cyclopentane analog, however, the corresponding distance is 2.6 Å. While this is still less than the sum of the van der Waals radii in *cis*-1-amino-1,3-dicarboxycyclopentane, and therefore some crowding would be expected, there should be significantly less steric hindrance with this substrate. At such short distances, the nonbonded energy is extremely sensitive to small changes, and a difference of 0.4 Å could therefore lead to a large decrease in this energy. These considerations suggest that amide formation from the cyclopentane analog would be more efficient than from either the cyclohexane analog or from *threo*-γ-methyl-L-glutamate. Such expectation is in accord with the data; thus, the relative V_{max} values for amide synthesis from L-glutamate, the cyclo-

[2] All calculations were performed using the computer program previously described (Gass, 1970) modified for use with a CDC 6400 computer. In calculating the coordinates of the molecule, the following parameters were assumed: C—C bond length, 1.54 Å; C—H, 1.1 Å; C—N, 1.47 Å; C=O, 1.21 Å; C—O, 1.43 Å. Angles between all substituents on the ring are 109° 28′; all carboxyl angles are 120°; C–C–C angles of the cyclopentane ring are 108° except as discussed in the text.

pentane analog, the cyclohexane analog, and *threo*-γ-methyl-L-glutamate are, respectively, 100, 43, 29, and 27. It may also be relevant that the K_m value for ammonia with the cyclopentane analog is about 10% of that found with the cyclohexane analog. These calculations and considerations seem to provide an explanation for the more effective utilization of ammonia with the cyclopentane analog; however, it is evident that additional investigations are required for full understanding of the significance of the observed kinetic parameters. The calculations show that the more puckered the ring, the more nearly the cyclopentane analog approaches the extended conformation of L-glutamate and therefore the better it fits to the previously calculated enzyme binding sites. While it is clearly of importance that the conformation of *cis*-1-amino-1,3-dicarboxycyclopentane ultimately be determined by independent methods, the present studies suggest the possibility that enzymatic approaches may be of value in conformational analysis.

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In Vitro Assembly of Aldolase. Kinetics of Refolding, Subunit Reassociation, and Reactivation†

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ABSTRACT: The kinetics of renaturation of aldolase, denatured in 6 M guanidine hydrochloride, were measured as a function of temperature, substrate (fructose 1,6-diphosphate), Gdn·HCl, and enzyme concentration. A comparative study was made of the *in vitro* rates of refolding, subunit reassociation, and reactivation. The major regain of secondary and tertiary structure, as determined by optical rotation, was complete within 30 sec of renaturation for temperatures between 0 and 25° and Gdn·HCl concentrations between 0 and 2 M. Dispersion curves for the renatured enzyme were nearly identical to that for native aldolase. The kinetics of subunit reassociation, as determined by Rayleigh light scattering, were biphasic—phase I an initial rapid association producing a mixture of monomers and dimers and phase II a further slow association leading to the formation of primarily tetramer. The rate of phase II reassociation increased with temperature and substrate concentration, decreased with Gdn·HCl concentration and was independent of initial enzyme concentration. Rates of regain of enzymatic activity closely paralleled phase II rates of reassociation under all renaturing conditions. Reactivation and reassociation kinetics were consistent with two or more rate-limiting sequential or parallel first-order reactions. Renaturation results are discussed in terms of an assembly mechanism for aldolase in which (1) dissociated and unfolded polypeptide chains both rapidly refold and partially reassociate to yield a mixture of structured, but inactive, monomers and dimers, (2) either or both of these species subsequently undergo a slow, first-order, minor conformational change, followed by (3) rapid association to tetramers with simultaneous regain of enzymatic activity.

Numerous studies have now indicated that many subunit proteins after complete disruption of their secondary, tertiary, and quaternary structure will spontaneously reassume a native or near native conformation simply upon removal of the denaturing conditions. In spite of these studies, however, still little is known about the mechanism, or sequence of events, leading from the dissociated and unordered polypeptide chain to the associated and uniquely structured subunit protein. A kinetic investigation of the *in vitro* renaturation of the subunit enzyme aldolase (fructose 1,6-di-

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