Stereospecific Synthesis of α-Methyl-L-glutamine by Glutamine Synthetase*

Herbert M. Kagan, Lois R. Manning, and Alton Meister

ABSTRACT: Although glutamine synthetase is active with both isomers of glutamic acid, the enzyme acts stereospecifically toward α-methylglutamic acid producing only a single α-methylglutamine isomer. α-Methyl-L-glutamine and both optical isomers of α-methylglutamic acid have been prepared.

Designation of the configuration of α-methyl-L-glutamine is supported by studies with *Escherichia coli* L-glutaminase, D-glutamic acid cyclotransferase, and *Azotobacter agilis* amidase. The last-mentioned enzyme catalyzes the hydrolysis of both L- and D-glutamine, but acts only on the L isomer of α-methylglutamine. Models of the substrates have been constructed and an explanation is proposed for the marked increase in stereospecificity associated with introduction of an α-methyl group.

Glutamine synthetase, which catalyzes the synthesis of glutamine from glutamic acid and ammonia according to the equation L-(or D-)glutamic acid + NH_3 + ATP $\rightleftharpoons$ L-(or D-)glutamine + ADP + P_i, also acts on a number of glutamate analogs and substituted glutamate derivatives to yield the corresponding amides and hydroxamates (Meister, 1962).[1] It is of particular interest that the enzyme is active with D-glutamic acid as well as L-glutamic acid; enzymatically synthesized D-glutamine has been isolated and characterized (Levintow and Meister, 1953, 1954). Although it has been reported that α-methyl-DL-glutamic acid is a substrate for glutamine synthetase (Braganca *et al.*, 1952; Lichtenstein *et al.*, 1953), the product formed was not isolated nor were studies with the individual optical isomers of this amino acid carried out. The present studies were stimulated by the observation that only about 50% of racemic α-methylglutamic acid was utilized by glutamine synthetase suggesting that, in contrast to its action on glutamic acid, the enzyme acts stereospecifically on α-methylglutamic acid to yield a single α-methylglutamine isomer. This suggestion has been substantiated by experiments in which one of the isomers of α-methylglutamine and both optical isomers of α-methylglutamic acid have been obtained. Evidence derived from studies with glutamine synthetase, D-glutamic acid cyclotransferase, and L-glutaminase indicates that the α-methylglutamine synthesized by glutamine synthetase is of the L-configuration.

Experimental

Materials. The nucleotides and sodium phosphoenolpyruvate were obtained from the Sigma Chemical Co. Pyruvate kinase and pronase were obtained from Calbiochem. α-Methyl-DL-glutamine was prepared as previously described (Meister, 1954). Glutamine synthetase from sheep brain (Pamiljans *et al.*, 1962), *Escherichia coli* L-glutaminase (Meister *et al.*, 1955), *Azotobacter agilis* amidase (Ehrenfeld *et al.*, 1963), rat kidney D-glutamic acid cyclotransferase (Meister *et al.*, 1963), and renal acylases I and II (Greenstein and Winitz, 1961) were prepared as described. Beef pancreas carboxypeptidase was obtained from the Worthington Biochemical Corp.

Methods. Glutamine synthetase activity was followed by hydroxamate and phosphate determinations as previously described (Pamiljans *et al.*, 1962). Ascending paper chromatography was carried out on Whatman No. 1 paper in solvents consisting of (a) 88% aqueous phenol–concd NH_4OH (99:1), and (b) 1-butanol–acetic acid–water (4:1:1). The R_F values for α-methylglutamic acid, α-methylglutamine, glutamic acid, and glutamine were, for (a), 0.34, 0.72, 0.20, and 0.58, and, for (b), 0.33, 0.26, 0.20, and 0.14, respectively.

α-Methylglutamic acid was determined by the ninhydrin method of Rosen (1957). Under the conditions described, α-methylglutamic acid gave about 25% of the color given by glutamic acid. Color development was considerably slower with α-methylglutamic acid than with glutamic acid, and it was found that maximum color was attained only after 30 minutes at 100°. Wilson and Snell (1962) have previously noted relatively slow development of color on treating α-methylserine with ninhydrin. In the present studies a heating time of 35 minutes was employed. The maximal color value for α-methylglutamic acid was 0.53 that for glutamic acid.

Results

Enzymatic Synthesis of α-Methylglutamine. When glutamine synthetase was incubated with α-methyl-

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate.

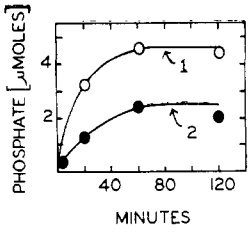

FIGURE 1: Course of amide synthesis from L-glutamic acid and α-methyl-DL-glutamic acid. The reaction mixtures contained imidazole-HCl buffer (*p*H 7.2, 50 μmoles), $MgCl_2$ (20 μmoles), 2-mercaptoethanol (25 μmoles), ATP (10 μmoles), NH_4Cl (100 μmoles), glutamine synthetase (44 units), and L-glutamic acid (curve 1) or α-methyl-DL-glutamic acid (curve 2) (5 μmoles) in a final volume of 1.0 ml; 37°.

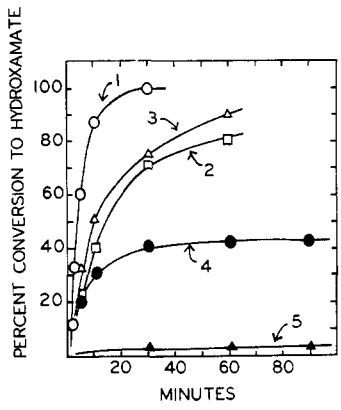

FIGURE 2: Synthesis of hydroxamates by glutamine synthetase. The reaction mixtures contained imidazole-HCl buffer (*p*H 7.2, 50 μmoles), $MgCl_2$ (20 μmoles), ATP (10 μmoles), 2-mercaptoethanol (25 μmoles), hydroxylamine hydrochloride (adjusted to *p*H 7.2 with sodium hydroxide; 100 μmoles), amino acid (1.0 μmole), and enzyme (43.2 units) in a final volume of 1.0 ml.; 37°. Curve 1, L-glutamic acid; curve 2, D-glutamic acid; curve 3, α-methyl-L-glutamic acid; curve 4, α-methyl-DL-glutamic acid; curve 5, α-methyl-D-glutamic acid.

DL-glutamic acid, ATP, Mg^{2+}, and NH_4Cl, the formation of phosphate proceeded to a value that approached 50% of that expected for complete reaction of the added amino acid (Figure 1). Experiments in which additional amounts of enzyme were added failed to increase the formation of inorganic phosphate beyond 50% of the theoretical. Under the same conditions, but with L-glutamic acid, more than 90% of the theoretical amount of inorganic phosphate was formed. Paper chromatographic study of reaction mixtures containing α-methyl-DL-glutamic acid after 60 minutes of incubation under the conditions described in Figure 1 revealed the disappearance of approximately half the substrate and formation of a new compound, which exhibited an R_F value identical to that of authentic α-methyl-DL-glutamine.

A large-scale experiment was carried out to facilitate isolation of both the enzymatically synthesized α-methylglutamine and the unsusceptible α-methylglutamic acid. In order to achieve as complete a reaction of the susceptible substrate as possible, the large-scale experiment was carried out in the presence of pyruvate kinase and phosphoenolpyruvate, and less than stoichiometric amounts of ATP.[2] The relatively small amount of nucleotide present also facilitated subsequent isolation of the amino acids. The reaction mixture contained α-methyl-DL-glutamic acid (2.254 g; 14 mmoles), ATP (1 mmole), $MgCl_2$ (2 mmoles), phosphoenolpyruvate (7.7 mmoles), NH_4Cl (10 mmoles), pyruvate kinase (7 mg; 917 units), 2-mercaptoethanol (150 μmoles), and glutamine synthetase (12 mg, 816 units) in a final volume of 31 ml. The reaction mixture was adjusted to *p*H 7.2 and incubated at 37° with gentle shaking. The formation of inorganic phosphate reached a value that was within experimental error of completion (i.e., 7 mmoles) after 7.5 hours; after a total of 8 hours of incubation, cold ethanol (100 ml) was added and the precipitated protein was removed by centrifugation and washed by centrifugation with 30 ml of 50% ethanol. The supernatant solutions were combined and concentrated at 25° in a flash evaporator to a thick oil weighing 2 g. This was dissolved in 10 ml of water and applied to the top of a column of Dowex 50 (H^+; 2 × 7 cm) at 4°. The column was washed with 140 ml of water and then eluted with 120 ml of 2 M NH_4OH. The water effluent contained virtually all of the phosphate, nucleotide, chloride, pyruvate, and 2-mercaptoethanol, while the amino acids were found in the NH_4OH effluent. The NH_4OH effluent was concentrated at 25° in a flash evaporator until a thick oil was obtained; this contained minimal amounts of ammonia as determined with Nessler's reagent. The oil was dissolved in 10 ml of water and chromatographed in two equal batches on a column of IRC-50 (H^+; 200-400 mesh; 90 × 2.4 cm); elution was carried out with water at 4°, and 175 fractions of 3.8 ml each were obtained. These were analyzed for α-methylglutamic acid and α-methylglutamine by paper chromatography. α-Methylglutamic acid was found in fractions 82–101; α-methylglutamine was found in fractions 104–150. Some nucleotide was present in fractions 58–74, but no chloride or ammonia was found in any of the fractions. Evaporation of the pooled amino acid and amino acid amide fractions followed by lyophilization gave 475 and 450 mg, respectively, of α-methylglutamic acid and α-methylglutamine.

Anal. (α-methylglutamic acid, mp 169–172°) Calcd for $C_6H_{11}O_4N$: N, 8.7. Found: N, 8.5. (α-methylglutamine, mp 166–167°) Calcd for $C_6H_{12}O_3N_2$: N, 17.5. Found: N, 17.2.

[2] A similar method was subsequently used for the isolation of enzymatically synthesized β-glutamine (Khedouri *et al.*, 1964).

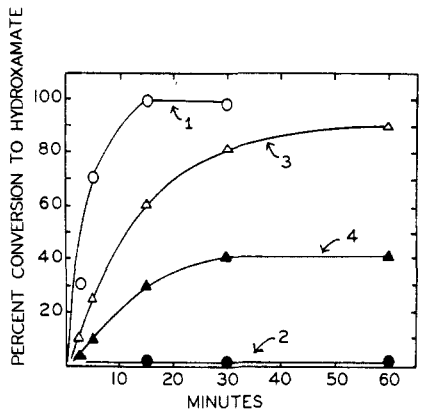

FIGURE 3: γ-Glutamyl transfer reactions. The reaction mixtures contained imidazole-HCl buffer (*p*H 7.2, 50 μmoles), $MnCl_2$ (5 μmoles), ADP (0.1 μmole), K_2HAsO_4 (*p*H 7.2, 25 μmoles), hydroxylamine hydrochloride (adjusted to *p*H 7.2 with sodium hydroxide, 100 μmoles), amino acid (2 μmoles), and enzyme (43.2 units), in a final volume of 1.0 ml. Curve 1, L-glutamine; curve 2, D-glutamine; curve 3, α-methyl-L-glutamine; curve 4, α-methyl-DL-glutamine.

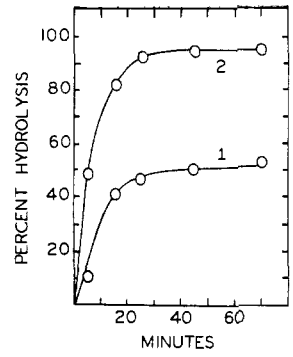

FIGURE 4: Hydrolysis of α-methyl-DL-glutamine and DL-glutamine by *A. agilis* amidase. The reaction mixtures contained imidazole-HCl buffer (25 μmoles, *p*H 7.2), enzyme (6.4 units), and α-methyl-DL-glutamine (3.6 μmoles) or DL-glutamine (3.3 μmoles) in a final volume of 0.5 ml. Curve 1, α-methyl-DL-glutamine; curve 2, DL-glutamine.

The products were homogeneous on paper chromatography. Because of the small amounts of material available, no attempt was made to crystallize the products. Acid hydrolysis of α-methylglutamine (3 N HCl, 120°, 30 minutes) gave stoichiometric amounts of ammonia and (after removal of ammonia by alkalinization and aeration) α-methylglutamic acid. The enzymatically synthesized α-methylglutamine and the α-methylglutamic acid derived from it are considered to be of the L configuration (*vide infra*).

Studies with Glutamine Synthetase and the Isomers of α-Methylglutamic Acid. When α-methyl-L-glutamic acid and α-methyl-D-glutamic acid, obtained as described, were incubated with glutamine synthetase, ATP, Mg^{2+}, and hydroxylamine, only α-methyl-L-glutamic acid (Figure 2, curve 3) was significantly active. Studies with L-glutamic acid (curve 1), D-glutamic acid (curve 2), and α-methyl-DL-glutamic acid (curve 4) were carried out for comparative purposes. Little, if any, hydroxamate was formed when α-methyl-D-glutamic acid was incubated under these conditions (curve 5). The very low activity observed with α-methyl-D-glutamic acid (1–2% in various experiments) probably may be ascribed to contamination with α-methyl-L-glutamic acid. Such contamination could be due to failure of the preparative enzymatic reaction to go to completion (cf. Levintow and Meister, 1954), or possibly to nonenzymatic hydrolysis of enzymatically synthesized α-methyl-L-glutamine.

Since it is known that the relative activities with L-glutamic acid and D-glutamic acid may vary depending upon *p*H and the nature and concentration of divalent metal ion, the synthesis of hydroxamate from α-methylglutamic acid was determined under various conditions. Studies were carried out with α-methyl-D-glutamic acid at *p*H values of 5.5, 7.2, and 7.8. Experiments at each of these values of *p*H were carried out with concentrations of Mg^{2+}, Mn^{2+}, and Co^{2+} varying from 0.005 to 0.1 M. In none of these experiments was more than 1.7% of the amino acid converted to the hydroxamate. In a similar series of studies with α-methyl-DL-glutamic acid no more than 48.3% of the added amino acid was converted to the hydroxamate. In other experiments carried out using conditions similar to those described in Figure 2, the effect of added α-methyl-D-glutamic acid on the formation of α-methyl-L-γ-glutamylhydroxamate from α-methyl-L-glutamic acid was determined. With concentrations of α-methyl-L-glutamic acid and α-methyl-D-glutamic acid of 0.0046 and 0.01 M, respectively, only 9% inhibition was observed.

α-Methyl-DL-glutamine and α-methyl-L-glutamine were examined as substrates for the γ-glutamyl transfer reaction. As indicated in Figure 3, under the conditions employed, about 90% of the added α-methyl-L-glutamine (curve 3) was utilized and about half of this amount of α-methyl-DL-glutamine (curve 4) reacted. In agreement with previous findings (Meister, 1962), the reaction with L-glutamine was rapid (curve 1) while little if any transfer was observed with D-glutamine (curve 2). The findings are consistent with the conclusion that α-methyl-D-glutamine is also not a very active substrate for the transfer reaction.

Other Enzymatic Studies. The action of *E. coli* L-glutaminase (Meister *et al.*, 1955) and *A. agilis* amidase (Ehrenfeld *et al.*, 1963) on α-methyl-L-glutamine and α-methyl-DL-glutamine was determined, using the conditions previously described. Both enzymes catalyzed the hydrolysis of α-methyl-L-glutamine virtually to completion and, within experimental error, 50% of α-methyl-DL-glutamine. In confirmation of previous findings *E. coli* glutaminase did not catalyze appreciable hydrolysis of D-glutamine, while the *A. agilis* enzyme

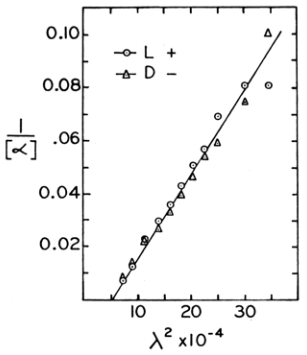

FIGURE 5: Optical rotatory dispersion curves of α-methyl-L-glutamic acid and α-methyl-D-glutamic acid. Concentrations: L-, 1.375%; D-, 2.01%; in 3 N HCl.

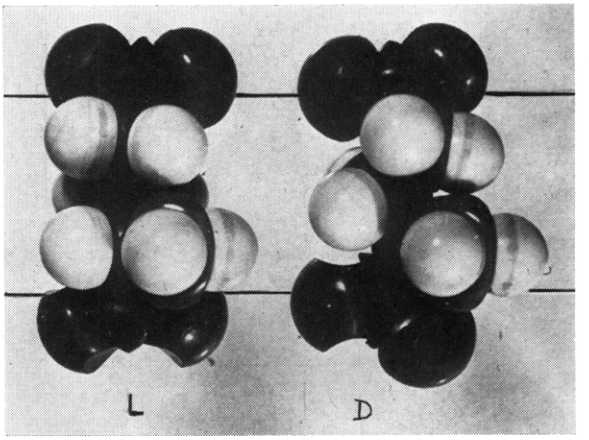

FIGURE 6: Models of L-glutamic acid and D-glutamic acid (see text).

hydrolyzed D-glutamine at about 60% of the rate observed for hydrolysis of L-glutamine. Under conditions in which the latter enzyme hydrolyzed DL-glutamine completely (Figure 4), the reaction appeared to reach a plateau when about half of the amide nitrogen of α-methyl-DL-glutamine was released as ammonia. The data suggest that the *A. agilis* enzyme does not hydrolyze α-methyl-D-glutamine at an appreciable rate.

Neither isomer of α-methylglutamic acid was found to be susceptible to L-glutamic acid decarboxylase prepared and studied as described (Meister *et al.*, 1951). *N*-Chloroacetyl-α-methyl-DL-glutamic acid was prepared (Greenstein and Winitz, 1961) and incubated with acylase I, acylase II, pronase, and carboxypeptidase in separate experiments; no hydrolysis of the *N*-chloroacetyl compound was observed as determined by the ninhydrin method (Rosen, 1957). Previous studies showed that both α-methyl-DL-glutamate and α-methyl-D-glutamate were substrates for D-glutamic acid cyclotransferase (Meister *et al.*, 1963). These observations have been confirmed and it was also shown that D-glutamic acid cyclotransferase does not act on α-methyl-L-glutamic acid.[3]

Optical Rotatory Dispersion of the Isomers of α-Methylglutamic Acid. These determinations were carried out with a Cary Model 60 recording spectropolarimeter at 25° in 3 *N* HCl with a 1-cm light path. A plot of the data based on a one-term Drude equation is given in Figure 5. Within experimental error (due largely to instrumental variability in the range of 500–600 mμ) the data indicate equal and opposite optical rotations over the spectral range examined. Specific optical rotations of +18.2°, +19.8°, +28.1°, and +43.6° were obtained, respectively, at 500, 450, 400, and 350 mμ for α-methyl-L-glutamic acid; the corresponding values for α-methyl-D-glutamic acid were −17.0°, −21.8°, −29.9°, and −43.4°.

Discussion

The data indicate that the action of glutamine synthetase on α-methylglutamic acid is specific for one optical isomer, and that such stereoselectivity can be utilized to effect the enzymatic resolution of α-methyl-DL-glutamic acid. The strict stereospecificity of the enzyme toward α-methylglutamic acid thus contrasts with its action on glutamic acid, the natural substrate. D-Glutamic acid is amidated less rapidly than L-glutamic acid, but there is evidence that both isomers are activated at substantially similar rates (Krishnaswamy *et al.*, 1962). In considering the substrate specificity of glutamine synthetase, it seems more remarkable that both optical isomers of glutamic acid are substrates than it does that only one isomer of α-methylglutamic acid is active. Although detailed information concerning the structure of the active site of glutamine synthetase is not yet at hand, a possible explanation for the observed specificity may be tentatively considered. It appears reasonable to assume that the enzyme has specific binding sites for the amino and carboxyl groups of L-glutamic acid, and that the respective functional groups of the other substrates combine with the same enzyme sites. Second, it may logically be postulated that the glutamic acid carbon chain is oriented on the enzyme surface in the fully (or almost fully) extended form so as to permit the maximum (or almost maximum) distance between the carboxyl groups. This postulate follows from the observation that aspartic acid is neither a substrate nor an inhibitor of the enzyme. If one constructs models of L- and D-glutamic acid in which the carbon chains are fully extended, it can be seen that it is possible to bring the respective amino and carboxyl groups to almost the same hypothetical enzyme binding sites (Figure 6). To effect such contacts with the enzyme would require that the β- and γ-carbon atoms of the isomers of glutamic acid occupy somewhat different

[3] We wish to thank Mr. Peter Polgar for assistance in these experiments. The formation of α-methylpyrrolidonecarboxylic acid (5-oxo-2-methylpyrrolidine-2-carboxylic acid) was determined by gas-liquid chromatography; the details of this procedure will be presented in a subsequent publication (P. Polgar and A. Meister, 1965, unpublished data).

positions; however, it is known that the enzyme acts on glutamic acid derivatives possessing β- and γ-substitutions (Meister, 1962), implying that an exact fit of this portion of the substrate to the enzyme is not necessary. Assuming this interpretation to be correct, one can arrive at a plausible explanation for the strict stereospecificity of the enzyme toward α-methylglutamic acid. Examination of the models shows that when the carboxyl and amino groups of L- and D-glutamic acids are brought as close as possible to the same respective hypothetical enzyme-binding sites, the α-hydrogen atoms of the glutamic acid isomers are oriented in markedly different ways. Thus replacement of the α-hydrogen atom of D-glutamic acid by a methyl group might be expected to exert a significantly different effect than such substitution of L-glutamic acid. Accordingly, the methyl group of α-methyl-D-glutamic acid would offer much greater steric hindrance to binding than that of its enantiomorph. For example, if the model of L-glutamic acid shown in Figure 6 is assumed to be on the enzyme surface, introduction of an α-methyl group would not be expected to interfere greatly with binding; on the other hand, replacement of the α-hydrogen atom of D-glutamic acid (on the under surface of the model shown in Figure 6) by a methyl group would displace the positions of the amino and carboxyl groups, and therefore interfere with attachment to the enzyme. Figure 7 shows models of α-methyl-L-glutamic acid and α-methyl-D-glutamic acid in which the respective methyl groups can be seen to be on opposite sides of the molecules; these models were constructed by replacing the α-hydrogen atoms of the models shown in Figure 6 with methyl groups. (The models have been turned about 75° to the left from the positions shown in Figure 6.)

Although the models constructed here suggest a way in which substitution of the α-hydrogen atom of D-glutamic acid could prevent its binding to the enzyme, while similar substitution of L-glutamic acid would not, other explanations for the experimental findings cannot be excluded and more evidence for the present hypothesis is needed. Any explanation proposed to account for the experimental findings described here must also take into account the interesting finding that β-glutamic acid is a substrate for the enzyme (Khedouri *et al.*, 1964) and that it is enzymatically converted to the D-isomer of β-glutamine. Extension of the considerations discussed above to β-glutamic acid and β-glutamine has led to conclusions which are consistent with the present ones (Khedouri and Meister, 1965).

The *A. agilis* amidase resembles glutamine synthetase in its specificity. Thus it hydrolyzes both isomers of glutamine, but cleaves only one isomer of α-methylglutamine (Figure 4). An explanation of the type suggested above for glutamine synthetase may also apply to the amidase.

Designation of the configuration of the isomer of α-methylglutamic acid that is amidated by glutamine synthetase as L is based entirely on the available enzymatic data. Thus glutamine synthetase amidates L-glutamic acid more rapidly than D-glutamic acid. *E. coli* glutaminase is highly specific for L-glutamine, and

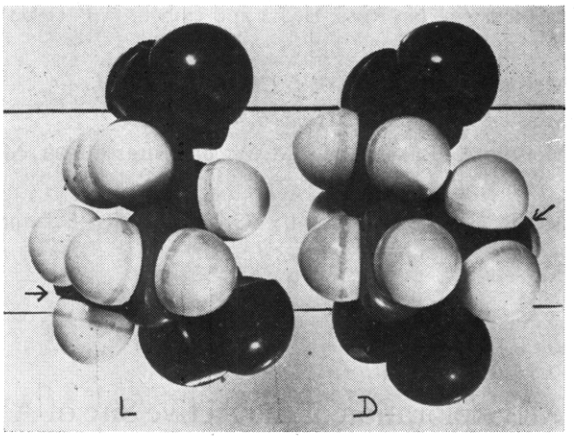

FIGURE 7: Models of α-methyl-L-glutamic acid and α-methyl-D-glutamic acid (see text). Arrows indicate the methyl carbon atoms.

this enzyme as well as the amidase from *A. Agilis* (which exhibits greater activity toward L-glutamine than D-glutamine) specifically hydrolyze the isomer of α-methylglutamine that is synthesized by glutamine synthetase. In addition, D-glutamic acid cyclotransferase, whose D-specificity has been demonstrated in studies on the isomers of glutamic acid and related substrates, acts only on the isomer of α-methylglutamic acid that is not amidated by glutamine synthetase. The designation of configurations of the α-methylglutamic acid isomers is based on the assumptions that the α-methyl group of enzymatically synthesized α-methylglutamine occupies the same position as the α-hydrogen atom of L-glutamic acid, and that the several enzymes studied here recognize α-methylglutamic acid as a derivative of glutamic acid. The isomer designated here as α-methyl-L-glutamic acid can also be considered as a derivative of D-alanine, and it is conceivable that an L-specific enzyme exists which would recognize this substrate as an L-alanine derivative and would therefore act on the enantiomorph of α-methyl-L-glutamic acid, i.e., α-(2-carboxyethyl)-L-alanine.

References

Braganca, B. M., Quastel, J. H., and Schucher, R. (1952), *Arch. Biochem. Biophys. 41*, 478.

Ehrenfeld, E., Marble, S. J., and Meister, A. (1963), *J. Biol. Chem. 238*, 3711.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, Wiley, p. 822.

Khedouri, E., and Meister, A. (1965), *J. Biol. Chem. 240* (in press).

Khedouri, E., Wellner, V. P., and Meister, A. (1964), *Biochemistry 3*, 824.

Krishnaswamy, P. R., Pamiljans, V., and Meister, A. (1962), *J. Biol. Chem. 237*, 2932.

Levintow, L., and Meister, A. (1953), *J. Am. Chem. Soc. 75*, 3039.

Levintow, L., and Meister, A. (1954), *J. Biol. Chem. 209*, 265.

Lichtenstein, N., Ross, H. E., and Cohen, P, P. (1953), *J. Biol. Chem. 201*, 117.
Meister, A. (1954), *J. Biol. Chem. 210*, 17.
Meister, A. (1962), *Enzymes 6*, 443.
Meister, A., Bukenberger, M. W., and Strassburger, M. (1963), *Biochem. Z. 338*, 217.
Meister, A., Levintow, L., Greenfield, R. E., and Abendschein, P. A. (1955), *J. Biol. Chem. 215*, 441.
Meister, A., Sober, H. A., and Tice, S. V. (1951), *J. Biol. Chem. 189*, 577, 591.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., and Meister, A. (1962), *Biochemistry 1*, 153.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Wilson, E. M., and Snell, E. E. (1962), *J. Biol. Chem. 237*, 3171.

An Exploration of the Active Site of Aldolase Using Structural Analogs of Fructose Diphosphate*

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ABSTRACT: A number of compounds which are structural analogs of fructose diphosphate were found to be competitive inhibitors of aldolase. It is concluded from comparison of the K_I (enzyme-inhibitor dissociation constant) values of these compounds that the binding of fructose diphosphate is primarily due to the phosphate groups, that hydroxyl groups do not contribute significantly to the binding, that the keto form is not bound preferentially, and that all forms of fructose diphosphate are acted upon by aldolase. The compounds and their K_I values are: D-arabinitol 1,5-diphosphate (1.5×10^{-6}); L-arabinitol 1,5-diphosphate (4.1×10^{-5}); xylitol 1,5-diphosphate (2.8×10^{-6}); ribitol 1,5-diphosphate (2.0×10^{-5}); 1,4-anhydro-DL-ribitol 5-phosphate (4.6×10^{-3}); 1,4-anhydro-DL-xylitol 5-phosphate (3.8×10^{-3}); 1,4-anhydro-D-arabinitol 5-phosphate (1.3×10^{-3}); D-glucitol 1,6-diphosphate (1.2×10^{-5}); 2,5-anhydro-D-mannitol 1,6-diphosphate (3.0×10^{-5}); 2,5-anhydro-D-glucitol 1,6-diphosphate (1.3×10^{-5}); ethylene glycol diphosphate (1.2×10^{-4}); 1,3-propanediol diphosphate (1.1×10^{-4}); 1,4-butanediol diphosphate (5.5×10^{-5}); 1,5-pentanediol diphosphate (2.9×10^{-5}); 1,6-hexanediol diphosphate (2.5×10^{-5}); 1,8-octanediol diphosphate (1.2×10^{-4}); 1,5-pentanediol-2-one diphosphate (3.0×10^{-5}); 3-hydroxytetrahydrofuran (3.6×10^{-2}). The synthesis of these compounds is described.

Ketohexose phosphates which are cleaved by aldolase[1] are fructose 1,6-diphosphate (Meyerhof, 1939), L-sorbose 1,6-diphosphate (Tung *et al.*, 1954; Richards and Rutter, 1961), D-fructose 1-phosphate (Tung *et al.*, 1954; Richards and Rutter, 1961), D-tagatose 1,6-diphosphate (Tung *et al.*, 1954), 5,6-dideoxy-D-fructose 1-phosphate (Lehninger *et al.*, 1955), and L-sorbose 1-phosphate (Tung *et al.*, 1954; Richards and Rutter, 1961). Of these the possibility must be considered that D-tagatose 1,6-diphosphate is cleaved by myogen A which was present in the assay medium and is not, therefore, a substrate for aldolase (Rutter, 1961).

The condensation of a number of aldehydes with dihydroxyacetone phosphate in the presence of aldolases has been observed and the products of such condensations presumably are substrates for the cleavage reaction, although their suitability as substrates relative to fructose 1,6-diphosphate has not been established (Rutter, 1961). Many of the materials which serve as substrates for the cleavage reaction and the normal substrate (fructose 1,6-diphosphate) can exist in carbonyl or hemiketal forms and the question arises, "Which form is the substrate?" The fact that certain compounds which can exist only as the keto form are cleaved by aldolase (Lehninger *et al.*, 1955; Rutter, 1961) does not remove the possibility that one or both of the hemiketal forms is preferentially bound and opened to the keto form prior to cleavage.

The study reported here was carried out in an attempt

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[1] This communication is concerned only with rabbit muscle aldolase.