

Activity of Glutamine Synthetase toward the Optical Isomers of α -Aminoadipic Acid*

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ABSTRACT: Purified sheep brain glutamine synthetase acts on the optical isomers of α -aminoadipic acid thus catalyzing the synthesis of L- and D-homoglutamine, the analogous ω -hydroxamic acids, and (in the absence of ammonia and hydroxylamine) the formation of 6-piperidone-2-carboxylic acid. Values for apparent K_m and relative maximal velocity were determined for the several reactions. Although the rates of hydroxamate formation from the α -aminoadipic acids are substantial (as compared to the isomers of glutamic acid), the rates of homoglutamine synthesis are less than 3% of those of hydroxamate formation.

Previous studies in this laboratory have led to the conclusion that L-glutamic acid and other substrates of glutamine synthetase are bound to the enzyme in an extended conformation in which the carboxyl carbon atoms are as far apart as possible¹ (Kagan *et al.*, 1965; Khedouri and Meister, 1965; Kagan and Meister, 1966a,b). These investigations have dealt with the explanation of the curious optical specificity of the enzyme toward various derivatives of glutamic acid. The hypothesis that the substrate is bound to the enzyme in an extended conformation is consistent with the available data on substrates containing carbon chains of five atoms and is also in accord with the fact that aspartic acid is neither a substrate nor an inhibitor of the enzyme.

It has been known for some time that glutamine synthetase from peas is active toward both optical isomers of α -aminoadipic acid (Levintow and Meister, 1953), and similar observations were subsequently made with the highly purified sheep brain enzyme (Krishnaswamy *et al.*, 1962). In an effort to elucidate further the action of glutamine synthetase on substrates with carbon chains longer than that of glutamic acid, we have reexamined the susceptibility of D- and L- α -aminoadipic acid to sheep brain glutamine

synthetase and have constructed models of the isomers of α -aminoadipic acid that permit consideration of the manner in which these substrates combine with the enzyme. These studies suggest that L- and D- α -aminoadipic acids attach to the active site of the enzyme in a modified extended conformation. It is striking that both isomers of α -aminoadipic acid react at appreciable rates with hydroxylamine but react very much more slowly with ammonia; an explanation for this finding is proposed in this paper.

Study of molecular models of α -aminoadipic acid and considerations derived from previous studies on substrates with carbon chains of five atoms have led to the conclusion that L- and D- α -aminoadipic acids attach to the active site of the enzyme in a modified extended conformation. It is proposed that the δ -methylene moiety of α -aminoadipic acid is oriented on the enzyme in such a manner as to hinder the attack of enzyme-bound ammonia on the activated ω -carboxyl carbon atom or the binding of ammonia to the enzyme, or both.

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Experimental Section

Materials. Glutamine synthetase was isolated from sheep brain as previously described (Pamijans *et al.*, 1962).² L- and D- α -aminoadipic acids were obtained from the corresponding racemate by enzymatic resolution (Greenstein *et al.*, 1953). DL- α -Aminopimelic acid was obtained from Nutritional Biochemicals Corp. Crystalline lactate dehydrogenase and pyruvate kinase were purchased from C. F. Boehringer and Soehn. DPNH³ and sodium phosphoenolpyruvate were obtained from Sigma Chemical Corp.

Methods. The initial rates of reaction with L- and D- α -aminoadipate in the presence of NH₄Cl were followed by determinations of inorganic phosphate (Fiske and Subbarow, 1925) or of ADP as described

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¹ The distance between the centers of the α - and γ -carboxyl carbon atoms of L-glutamic acid is estimated to be 5 Å from measurements of Dreiding models and from drawings prepared by Mr. Jerald D. Gass of this laboratory.

² A unit of glutamine synthetase activity is defined as that amount of enzyme which catalyzes the synthesis of 1 μ mole of γ -glutamylhydroxamic acid under the standard conditions previously described (Wellner and Meister, 1966).

³ Abbreviations used: ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide; ADP, adenosine diphosphate.

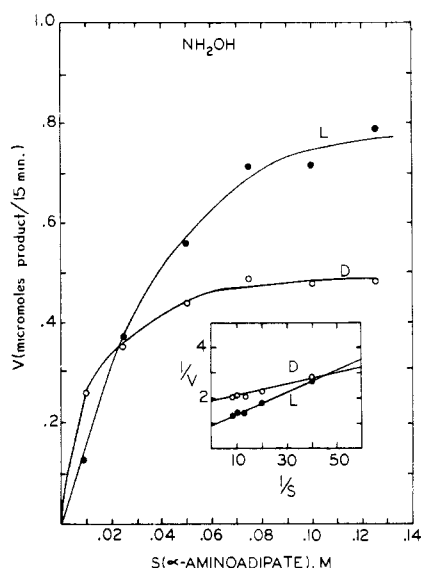


FIGURE 1: Effect of α -aminoadipate concentration on activity (with hydroxylamine). The reaction mixtures consisted of imidazole-HCl buffer (pH 7.2, 50 μ moles), MgCl_2 (20 μ moles), 2-mercaptoethanol (25 μ moles), ATP (10 μ moles), NH_2OH (pH 7.2, 100 μ moles), glutamine synthetase (5 units), and sodium L- or D- α -aminoadipate in a final volume of 1.0 ml, 37°.

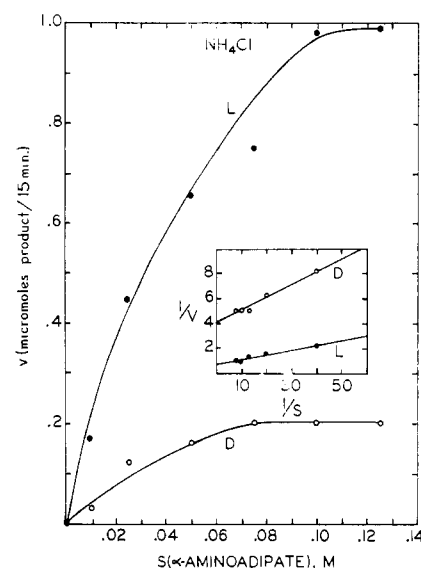


FIGURE 2: Effect of α -aminoadipate concentration on activity (with ammonium chloride). The reaction mixtures consisted of imidazole-HCl buffer (pH 7.2, 50 μ moles), MgCl_2 (20 μ moles), 2-mercaptoethanol (25 μ moles), ATP (10 μ moles), NH_4Cl (100 μ moles), glutamine synthetase (200 units), and sodium L- or D- α -aminoadipate in a final volume of 1.0 ml, 37°.

below. The reactions with NH_2OH were followed by the hydroxamate assay as previously described using a value of absorbancy at 535 $m\mu$ of 0.340/ μ mole (Wellner and Meister, 1966). The cyclization reactions were followed by determinations of ADP formation carried out in a coupled system containing pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, and DPNH. The reaction mixtures consisted of Tris-HCl buffer (pH 7.6, 50 μ moles), KCl (75 μ moles), MgCl_2 (20 μ moles), lactic dehydrogenase (3 units), pyruvate kinase (3 units), phosphoenolpyruvate (3.3 μ moles), DPNH (0.3 μ mole/ml), ATP (10 μ moles), glutamine synthetase, and sodium D- or L- α -aminoadipate in a final volume of 0.7–1.4 ml, 37°. The decrease in absorbancy at 340 $m\mu$ was followed with a Cary Model 11 recording spectrophotometer.

Results

Activity of Glutamine Synthetase toward α -Amino-adipic Acid and Other Dicarboxylic Acids. In confirmation of earlier studies (Levintow and Meister, 1953; Krishnaswamy *et al.*, 1962) it was found that both optical isomers of α -aminoadipic acid are substrates of the enzyme. The homoglutamine formed in reaction mixtures containing L- and D- α -aminoadipic acids and NH_4Cl was identified by paper chromatography; thus, in a solvent consisting of phenol- H_2O - NH_4OH (80:20:1) the R_F values for α -aminoadipic acid and homoglutamine were, respectively, 0.32 and 0.69. Previous findings of enzymatic hydroxamate formation

from NH_2OH and α -aminoadipic acid and of the cyclization of α -aminoadipic acid to 6-piperidone 2-carboxylate were also confirmed.

In studies carried out with relatively high concentrations of enzyme (190 units/ml) and substrate (0.125 M), no activity was observed toward L-aspartic acid or α -aminomalonic acid. On the other hand, evidence was obtained that the enzyme exhibits slight but detectable activity toward DL- α -aminopimelic acid. Thus, with 0.05 M DL- α -aminopimelic acid in the standard assay system containing hydroxylamine (Wellner and Meister, 1966) and 160 units/ml of glutamine synthetase, hydroxamate formation was readily detectable; the rate of this reaction was 0.29% of that observed with L-glutamic acid under comparable conditions. Under the same conditions, there was no detectable activity in the presence of ammonia and α -aminopimelate as judged by determinations of inorganic phosphate. Paper chromatography revealed the formation of a FeCl_3 -reactive compound that differed in mobility from γ -glutamyl hydroxamate and δ -(α -aminoadipyl) hydroxamate. The hydroxamates enzymatically formed from glutamic acid, α -aminoadipic acid, and α -aminopimelic acid exhibited R_F values of 0.36, 0.39, and 0.54, respectively, in a solvent consisting of phenol- H_2O - NH_4OH (90:10:1); the corresponding values with a solvent consisting of *t*-butyl alcohol-acetic acid- H_2O (70:15:15) were 0.06, 0.07, and 0.10.

Kinetic Studies with the Isomers of α -Aminoadipic Acid. The effects of α -aminoadipate concentration

TABLE I: Values for Apparent K_m and Relative Maximal Velocity.^a

Reaction	L- α -Aminoadipate		D- α -Aminoadipate	
	Rel V_{max}^b	$K_m \times 10^3$ (M)	Rel V_{max}^b	$K_m \times 10^3$ (M)
δ -(α -Aminoadipyl)hydroxamate synthesis	22	48	11	14
Homoglutamine synthesis	0.63	50	0.19	26
6-Piperidone 2-carboxylate formation	0.050	100	0.033	33

^a Obtained by the method of Lineweaver and Burk (1934). The concentrations of NH_4Cl and NH_2OH were 0.1 M; other experimental details are as given in Figure 1. ^b Relative to a value of 100 for L-glutamate (with NH_4^+ or NH_2OH); see also Kagan and Meister (1966a,b).

on the rates of reaction with hydroxylamine and with ammonia are described in Figures 1 and 2. The data presented in Figure 2 were obtained with 40 times as much enzyme as used in the experiments described in Figure 1. It is therefore apparent that there is much more activity in the presence of hydroxylamine than with ammonia. D- α -Aminoadipate was much less reactive with ammonia than L- α -aminoadipate at all concentrations of amino acid studied. On the other hand, in the presence of hydroxylamine D- α -aminoadipate was about twice as active as L- α -aminoadipate at a substrate concentration of 0.01 M, about equally active at a substrate concentration of 0.02 M, and about one-half as active at relatively high substrate concentrations. There is generally less difference between the reactivity of the optical isomers of α -aminoadipic acid with hydroxylamine than with ammonia. The values for K_m and relative maximal velocity obtained from these data are summarized in Table I. Although the K_m values do not differ greatly from each other, the values for relative maximal velocity are considerably lower with ammonia than with hydroxylamine. The ratios of the values for relative maximal velocity with hydroxylamine to those for ammonia are 35 and 58 for L- and D- α -aminoadipate, respectively. These compare with ratios of 1.0 (L-glutamate), 2.0 (D-glutamate), 0.9 (α -methyl-L-glutamate), 21 (*threo*- β -methyl-D-glutamate), 2.3 (*threo*- γ -methyl-L-glutamate), and 2.6 (β -glutamate) (Kagan and Meister, 1966a,b).

The K_m values observed in studies on the cyclization reactions are not far from those found in the presence of ammonia or hydroxylamine, while the rates of cyclization are, as noted previously (Krishnaswamy *et al.*, 1962), much lower than those for the synthesis reactions. The K_m for D-glutamate (cyclization) was determined in the same manner for comparative purposes; a value of 17×10^{-3} M was obtained (compared to 13×10^{-3} M and 3.8×10^{-3} M for the reactions with ammonia and hydroxylamine, respectively). The relative maximal velocity of the cyclization of D-glutamate was found to be 0.068 and is thus similar to those given in Table I for the cyclization of α -aminoadipate.

TABLE II: Apparent K_m Values for NH_4Cl and NH_2OH .^a

Amino Acid Substrate	$K_m \times 10^3$ (M)	
	NH_3	NH_2OH
L- α -Aminoadipate	57	33
D- α -Aminoadipate	48	36
L-Glutamate	0.18	0.15
D-Glutamate	14	3.5

^a Obtained by the method of Lineweaver and Burk (1934). The concentrations of α -aminoadipate and glutamate were, respectively, 0.15 and 0.05 M; other experimental details are as given in Figure 1.

The K_m values for ammonia and hydroxylamine determined in the presence of α -aminoadipate and glutamate are given in Table II. Although there was appreciable activity when methylamine was used in place of am-

TABLE III: Comparative Rates of Reaction with Ammonia, Hydroxylamine, and Methylamine.

Substrate	Rel V_{max}^a		Rel Act. ^b	
	NH_2OH	NH_3	NH_2OH	CH_3NH_2
L-Glutamate	100	100	94	82
D-Glutamate	54	27	41	2.0
L- α -Aminoadipate	22	0.63	23	<0.07
D- α -Aminoadipate	11	0.19	12	<0.07

^a From Kagan and Meister (1966b) and present paper (Table I). ^b Activity/100 units of enzyme in the standard assay system containing 0.1 M NH_2OH or 0.55 M CH_3NH_2 and 0.1 M amino acid substrate.

monia with the isomers of glutamate, there was no detectable reaction with either L- or D- α -aminoadipate in the presence of methylamine (Table III).

Discussion

It seems significant that both isomers of α -aminoadipate exhibit substantial activity in hydroxamate synthesis, but that they are less than 3% as active in homoglutamine synthesis. This difference in reactivity is remarkable when considered in relation to the results obtained with other substrates of glutamine synthetase. Thus, with the exception of *threo*- β -methyl-D-glutamate (which is 5% as active with ammonia as with hydroxylamine) all of the other substrates exhibit relative maximal velocity values with ammonia that are more than about 40% of the corresponding values with hydroxylamine. Although it does not seem possible at this time to provide cogent detailed explanations for all of the differences between the values of the kinetic constants that have been obtained with the various substrates of the enzyme, it seems pertinent to consider here the findings on the reactivity of α -aminoadipate with ammonia and hydroxylamine especially in relation to the hypothesis (Khedouri and Meister, 1965; Kagan *et al.*, 1965; Kagan and Meister, 1966a,b) concerning the conformation of enzyme-bound glutamic acid.

The ability of α -aminoadipic acid to serve as a substrate suggests that the amino and carboxyl groups of this molecule can attach to the enzyme at the same respective sites as those of L-glutamic acid and other substrates that possess a carbon chain of five atoms. Study of a model of L- α -aminoadipic acid has shown that it is possible to orient the carboxyl carbon atoms in space in a manner very similar to those of L-glutamic acid. To do this, a model of the fully extended form of L- α -aminoadipic acid is rotated between the γ - and δ -carbon atoms so as to bring the center of the ω -carboxyl carbon atom to a position which is very close to 5 Å from the center of the α -carboxyl carbon atom.¹ Figure 3 permits a comparison to be made between the model of L-glutamic acid presented previously (Kagan and Meister, 1966a) and the proposed conformation of L- α -aminoadipic acid. The γ -hydrogen, β -hydrogen, α -hydrogen, and α -amino nitrogen atoms of both models are in similar positions relative to the α -carboxyl group. The close similarity in the relationship between the carboxyl groups of the two models is evident from Figure 3 (B and C). On the other hand, it is apparent that the δ -methylene moiety of α -aminoadipic acid occupies a position corresponding in space to that of the ω -carboxyl group of L-glutamic acid; the ω -carboxyl group of L- α -aminoadipic acid is thus displaced downward in Figure 3A as compared to L-glutamic acid. This relationship can be more clearly seen in Figure 3C.

In an earlier communication from this laboratory it was suggested that the ammonia binding site on the enzyme lies just above the ω -carboxyl carbon atom of L-glutamic acid (see Figure 2A, Kagan and Meister,

1966b). It was also postulated that although ammonia attacks the activated carboxyl carbon atom from a site on the enzyme, hydroxylamine can attack directly without prior attachment to the enzyme. The lower reactivity of D-glutamic acid with ammonia as compared to hydroxylamine was ascribed to an unfavorable orientation of the carboxyl carbon atom in relation to the ammonia binding site on the enzyme. The possibility of steric hindrance of the attack by enzyme-bound ammonia on the activated carboxyl carbon atom was also considered as an explanation for the relatively decreased reactivity with ammonia of *threo*- γ -methyl-L-glutamic acid and *threo*- β -methyl-D-glutamic acid. Since the δ -methylene moiety of L- α -aminoadipic acid in the model proposed here is in about the same position (relative to C₁-C₄) as the ω -carboxyl group of L-glutamic acid, this group of the substrate might therefore be expected to interfere with the attack of ammonia from the enzyme. Furthermore, the possibility must be considered that the δ -hydrogen atoms of L- α -aminoadipic acid interfere with binding of ammonia to the enzyme. The finding that the apparent K_m values for ammonia with α -aminoadipate are higher than those for ammonia with glutamate is consistent with, but does not prove, that the affinity of the enzyme for ammonia is reduced in the presence of α -aminoadipate.

We have also constructed a model of D- α -aminoadipic acid following the general considerations adopted with the L isomer. A comparison of this model with that of D-glutamic acid previously presented (Kagan and Meister, 1966a) is given in Figure 3 (D and E). In analogy with the respective L isomers, there is close correspondence between the relative positions of the carboxyl groups, and the δ -hydrogen atoms of D- α -aminoadipic acid are in about the same relationship to the δ -carboxyl group as in L- α -aminoadipic acid. When models of α -aminopimelic acid were studied it was found possible to rotate the carbon chain as carried out for α -aminoadipic acid thus bringing the carboxyl and amino groups to the same relative positions as those of L-glutamic acid; to accomplish this there must be considerable displacement of the carbon chain in the region of the γ -carbon atom of L-glutamic acid. Such an effect would be expected to provide considerable steric hindrance to the binding of this substrate to the enzyme and would be consistent with the very low rate of activity observed.

It is of note that although methylamine is active with glutamic acid (leading to synthesis of the corresponding γ -N-methylamide), this ammonia analog is inactive with α -aminoadipic acid. This result suggests that methylamine may not effectively bind to the enzyme in the presence of α -aminoadipic acid, or if bound is very greatly hindered in its attack on the activated carboxyl carbon atom. Investigations with other ammonia analogs and the various amino acid substrates of the enzyme are in progress; such studies may provide further information concerning relationships between the binding of ammonia and glutamate to the enzyme.

FIGURE 3: Stereophotographs of models of L- α -amino adipic acid (right) and L-glutamic acid (left). (A) Comparison of models to show relationship of α and γ substituents to α -carboxyl groups. (B) View of the undersurfaces of the models shown in A; (C) Side view (from right side) of A. (D) Stereophotographs of models of D- α -amino adipic acid (right) and D-glutamic acid (left) oriented in a manner corresponding to A. (E) View of the undersurfaces of the models shown in D. To obtain a three-dimensional effect, place a mirror in the narrow space between each pair of photographs with the mirrored surface facing to the left. Then, look down from the top edge of the mirror (tilting the mirror toward the right as needed) so that the reflection of the photograph on the left is seen by the left eye and the photograph on the right is seen by the right eye. A three-dimensional view is seen when the two images coincide. (Both photographs should be about equally illuminated. A slightly sharper picture is obtained with a front-surfaced mirror than with the usual type of glass mirror.)



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Reaction Kinetics of the Ferrimyoglobin Azide System*

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ABSTRACT: Rate and equilibrium constants for azide binding by whale ferrimyoglobin have been determined and interpreted over essentially the entire pH range within which the myoglobin and its azide complex are stable. Kinetics of azide ferrimyoglobin (sperm whale) reactions measured over the range pH 4.5-7.0 gave the same rate constants at each pH whether determined by flow or relaxation techniques. Rates of formation and dissociation of the azide complex are

highly pH dependent in this range. The dependence coincides with about a 300-fold preferential binding of hydrazoic acid ($k = \sim 10^6 \text{ M}^{-1} \text{ sec}^{-1}$) compared to azide ion.

In the range pH 7-10 rates determined by stopped flow showed much less sensitivity to pH. Binding is comparatively slower for freshly redissolved whale myoglobin crystals but faster for redissolved crystalline seal myoglobin.

Early studies on the binding of azide by ferrimyoglobin (Kiese and Kaeske, 1942) showed the system to follow a simple mass action law. It was not clear from this work, however, whether one form of the ligand was bound preferentially. Among other uncharged Bronsted acids and their conjugate bases that are known to act as ligands with hemoproteins, particularly ferrimyoglobin, fluoride is reported to be very much less reactive than hydrofluoric acid (George and Hanania, 1954) whereas cyanide (Coryell *et al.*, 1937; Chance, 1952) and phenolate ions (George *et al.*, 1961) are said to bind somewhat faster than their conjugate acids.

In some cases the conclusions concerning relative reactivities are based on equilibrium studies and in

others on kinetic experiments. Reconciliation of results from both approaches has generally been difficult although Goldsack *et al.* (1965, 1966) have lately accomplished this to a limited extent with azide, cyanate, and hydrogen sulfide. To elucidate some details of ligand binding to ferrimyoglobin we have utilized equilibrium measurements as well as the intrinsically different kinetic techniques of stopped-flow and chemical relaxation in a study of the well-known azide ferrimyoglobin reaction.

Though the reaction of azide with crystalline metmyoglobin is much slower, and hence possibly quite different, than with dissolved metmyoglobin (Chance *et al.*, 1966), the over-all process in the solid state may be clearly pictured from X-ray diffraction results (Stryer *et al.*, 1964) to consist of a net isomorphous replacement of water by hydrazoic acid at the sixth coordination position of the heme iron(III) ion.

Experimental Section

Reagents. Solubilized sperm whale skeletal muscle

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