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INTERACTION OF SOLUBLE PIG HEART GLUTAMATE-ASPARTATE TRANSAMINASE WITH VARIOUS β,γ-UNSATURATED AMINO ACIDS

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SUMMARY - β-Ethylidene-DL-aspartate (βEA) and β-methylene-DL-glutamate (βMG) were synthesized and tested as potential suicide inhibitors of soluble pig heart glutamate-aspartate transaminase (sGAT). βMG was found to be a) a substrate with a very low turnover number relative to glutamate and b) a competitive inhibitor with respect to aspartate (albeit with a large binding constant). At high concentrations βMG inactivated the enzyme but only very slowly. βEA was also found to be a substrate with a very low turnover number; it did not inactivate the enzyme (1 hr, 25°C) even at a high concentration. However, βEA was found to bind to the enzyme with an affinity comparable to that of aspartate and glutamate. β-Methylene-DL-aspartate (βMA) has been shown to rapidly inactivate glutamate-aspartate transaminase. Therefore, it appears that glutamate-aspartate transaminase can bind analogues of aspartate with alkene groups in the β position. The conjugated carbonyl groups of βMA and βEA will enhance Michael addition in comparison with that expected for vinylglycine. On the other hand, the presence of the methyl groups should reduce the electrophilicity of the double bond of βEA compared to βMA. This deactivation and/or steric hindrance to Michael attack may account for the inability of βEA to inactivate sGAT. Therefore, it may be possible to design selective suicide inhibitors of glutamate-aspartate transaminase with the following structure: $HO_2CC(=CHX)CH(CO_2H)NH_2$, where X is an electron-withdrawing group. Ideally, X would increase the reactivity of the double bond while affording a minimum of steric hindrance to susceptible enzyme-bound bases.

Previous work from our laboratories has shown that β-methylene-DL-aspartate (βMA) is a reasonably selective irreversible inhibitor of soluble pig heart

Abbreviations used: βMA, β-methylene-DL-aspartate; βEA, β-ethylidene-DL-aspartate; βMG, β-methylene-DL-glutamate; βMAsn, β-methylene-DL-asparagine; βMGln, β-methylene-DL-glutamine; sGAT, soluble glutamate-aspartate transaminase; GAD, glutamate decarboxylase.

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glutamate-aspartate transaminase (sGAT) (1,2). The compound was later used to inhibit GAT in rat brain slices. These studies suggested an important role of the malate-aspartate shuttle for the translocation of "reducing equivalents" between cytosol and mitochondrion in rat brain (3). We presented evidence that βMA is a suicide inhibitor of sGAT and suggested that inactivation occurs by Michael addition of an enzyme-bound base to the β-methylene carbon of the conjugated ketimine intermediate(1,2). In agreement with this idea, Arnone **et al.** noted nearly quantitative release of pyridoxamine 5'-phosphate upon denaturing the βMA-inactivated α-subform of sGAT(4). Although βMA had no effect on two flavin-linked enzymes and on several pyridoxal 5'-phosphate-containing enzymes, βMA was shown to inhibit glutamate decarboxylase (GAD) in rat brain slices and homogenates (2,3), and rat liver cysteinesulfinate decarboxylase **in vitro** and **in vivo** (5). Although βMA had no effect on purified pig heart glutamate-alanine transaminase, βMA did inhibit this enzyme in crude rat liver homogenates (2). As a first step toward the design of a more specific inhibitor of sGAT we tested the effects of β-methylene-DL-glutamate (βMG) and β-ethylidene DL-aspartate (βEA) on purified pig heart sGAT, a number of other pyridoxal 5'-phosphate--containing enzymes and on D-and L-amino acid oxidases. This paper reports our findings.

MATERIALS AND METHODS

Enzymes and enzyme assays. L-Amino acid oxidase (**Crotalus Adamanteus**; 4.3 U/mg) and D-amino acid oxidase (hog kidney, 173 U/mg) were obtained from Sigma Chemical Company. Lactate dehydrogenase (bovine heart; 425 U/mg crystalline suspension in 2.2 M ammonium sulfate) was obtained from Worthington. Soluble pig heart glutamate-aspartate transaminase (pig heart; 200 U/mg in 3.2 M ammonium sulfate), glutamate-alanine transaminase (pig heart; 80 U/mg in 3.2 M ammonium sulfate), malate dehydrogenase (pig heart; 1200 U/mg in 50% glycerol) were obtained from Boerhinger Mannheim. Soluble glutamine transaminase L (rat liver; 4.0 U/mg in 30% glycerol), soluble glutamine transaminase K (rat kidney; 5.7 U/mg in 30% glycerol), and asparagine transaminase (rat liver; 0.3 U/mg in 30% glycerol) were purified as described (6-8), except that the L-albizzin-α-keto-γ-methiolbutyrate transaminase assay (9) was used during the purification of glutamine transaminase L. Tyrosine transaminase was purified from rat liver (following induction by intraperitoneal injection of dexamethasone in gelatin(10)) through the DEAE cellulose step of Hargrove **et al.** (11) (80 U/mg in 30% glycerol, 0.1 mM pyridoxal 5'-phosphate, 5 mM 2-mercaptoethanol). GABA transaminase and glutamate decarboxylase activities were measured in rat brain homogenates according to the method of Jung **et al.** (12). All other enzyme activities were measured as previous described (2,7-9). Saturated amino acid and α-keto acid substrates were obtained from Sigma Chemical Company.

Unsaturated amino acids. β-Methylene-DL-aspartate was made by the method of Dowd and Kaufman (13). β-Ethylidene-DL-aspartate was synthesized as follows: ethyl α-ketobutyrate was synthesized using the general method of Eliel and Hartmann(14) developed for the synthesis of α-keto acid ethyl esters. Thus, ethyl diethoxyacetate, prepared by the method of Moffett (15), was reacted with 1,3-propanedithiol (Aldrich Chemical Company) in BF_3-etherate yielding ethyl 1,3-dithiane-2-carboxylate. Alkylation of the dithiane anion with ethyl chloride yielded ethyl 2-ethyl-1,3-dithiane-2-carboxylate. The protecting 1,3-propanedithiol group was removed by oxidative hydrolysis with N-bromosuccinimide (14,16), yielding ethyl α-ketobutyrate which was converted to 1,1,2-tricarbethoxy-1-butene by a malonic ester synthesis (17). The triester was converted to β-ethylidene-DL-aspartate* using the method of Dowd and Kaufman (13) described in the synthesis of β-methylene-DL-aspartate. β-Ethylidene-DL-aspartate was analyzed as the monosodium salt monohydrate. Calculated for $C_6H_8NO_4Na \cdot H_2O$: C, 36.19; H, 5.05; N, 7.03. Found: C, 36.46; H, 4.78; N, 6.91.

β-Methylene-DL-glutamate, β-methylene-DL-glutamine and β-methylene-DL-asparagine were synthesized as described (18). Each of the unsaturated amino acids exhibited a single ninhydrin-positive spot on ascending paper chromatography in the following system: methyl ethyl ketone: **tert**-butyl alcohol: formic acid: H_2O (40 : 30 : 15 : 15 v/v) (19). R_f values in parentheses follow: aspartate (0.31), β-methyleneaspartate (0.39), β-ethylideneaspartate (0.43), glutamate (0.46), β-methyleneglutamate (0.51), glutamine (0.30), β-methyleneglutamine (0.63), asparagine (0.41), β-methyleneasparagine (0.48). The aspartate and glutamate analogues are stable, but the asparagine and glutamine derivatives were unstable in aqueous solution above pH 8.0, generating strongly UV-absorbing products within 30 minutes.

βMG and βEA as substrates of sGAT. sGAT (100 μg) was incubated in a 0.1 ml reaction mixture containing 50 mM βMG (or βEA), 15 mM oxaloacetate and 200 mM potassium phosphate at pH 7.2 for 2-10 h at 25°C. An aliquot (10 μl) was spotted on paper. After development (see above), the aspartate spot was compared to 1) that obtained from a reaction mixture without enzyme (non-enzymatic transamination) and 2) those obtained from mixtures containing 50 mM L-glutamate in place of unsaturated amino acid and 0, 0.01, 0.1, 1.0 and 10 μg of sGAT.

Determination of kinetic constants. Michaelis constants were determined from double reciprocal plots. The inhibition constant for inactivation of glutamate-aspartate transaminase by βMA was obtained from a plot of the reciprocal of the pseudo-first order rate constant of inactivation versus 1/[I] (2). βMG and βEA were both found to be competitive inhibitors with respect to aspartate in the reaction catalyzed by glutamate-aspartate transaminase, i.e. each plot of 1/V versus 1/S in the presence of a fixed inhibitor concentration intersected the 1/V axis at the same point. The inhibitor constants were determined from a replot of the slope versus inhibitor concentration (20).

RESULTS

Interaction of soluble pig heart glutamate-aspartate transaminase (sGAT) with β,γ-unsaturated amino acids. The results of the experiments are summarized in Table 1. The affinities of the enzyme for β-methylene-DL-aspartate and β-ethylidene-DL-aspartate are roughly comparable to the natural substrates, glu-

*This compound has been prepared independently by O. W. Griffith (Cornell University Medical College) in better yield starting with ethyl α-bromobutyrate and diethyl malonate. The compound prepared by this method is indistinguishable from that prepared by our procedure.

TABLE 1

Interaction of sGAT with Amino Acid Substrates and β,γ-Unsaturated Analogues[1]

Compound	Interaction	Kinetic parameters (mM)	References
L-Aspartate	Substrate	Absol. K_m=3.9, 0.9 App. K_m=5.4	21,22 present work
L-Glutamate	Substrate	Absol. K_m=8.9, 4.0	21,22
β-Methylene-DL-aspartate	Rapid suicide inhibition maximum $t_{1/2}$~1.8 min, 25°C	K_i=6.0	2
β-Ethylidene-DL-aspartate	No inactivation at 70 mM; substrate with low turnover rate;[2] Competitive inhibitor with respect to aspartate	App. K_i=15.4	present work
β-Methylene-DL-glutamate	Substrate with low turnover rate;[2] slow rate of inactivation ($t_{1/2}$~70 min; 70 mM); Competitive inhibitor with respect to aspartate	App. K_i= ~200[3]	present work

[1] In the present work all incubations were carried out in the presence of 100 mM potassium phosphate buffer, pH 7.2, 25°C (except where noted). Where turnover was measured, 10 mM α-ketoglutarate was the α-keto acid substrate.

[2] ~0.02% the rate exhibited with L-glutamate as substrate (in the presence of 200 mM potassium phosphate buffer, pH 7.2, 25%C).

[3] This value is approximate because the maximum concentration of βMG in the assay mixture is 100 mM.

tamate and aspartate. In a further experiment, incubation of sGAT with 10 mM βMA in 100 mM potassium phosphate buffer (pH 7.2) for 15 min at 25°C resulted in 86% loss of activity compared to a control lacking βMA. When 60 mM βEA was also included in the incubation mixture, the loss of activity was only 11%. The affinity of the enzyme for βMG is much lower than that for either βMA or βEA. βMG is, however, a substrate with a low turnover number. At high concentration (70 mM), βMG slowly inactivates sGAT in a pseudo first-order manner. The inactivation could not be reversed by addition of 0.1 mM pyridoxal 5'-phosphate or by dialysis. Qualitative paper chromatographic analysis of the deproteinized inactivated enzyme solutions revealed the presence of pyridoxamine 5'-phosphate (as noted previously (2) for βMA-inactivated sGAT). Neither β-methylene-DL-

asparagine (βMAsn) nor β-methylene-DL-glutamine (βMGln) were substrates for sGAT. βMAsn (70 mM, pH 7.2, 25°C) very slowly inactivated the enzyme ($t_{1/2}$~11 h); βMGln (70 mM) had no effect.

Interaction of other enzymes with β,γ-unsaturated amino acids. βMA, βMG, βMAsn and βMGln (70 mM, pH 7.2, 25°C; 1 h) had no effect on the activity of pig heart glutamate-alanine transaminase. No evidence could be found that these compounds were substrates (rate < 1% relative to that with glutamate), and βMG was found to be a competitive inhibitor with respect to alanine but with a relatively high binding constant (appK_m alanine = 30 mM; appK_i βMG ~ 200 mM; in the presence of 10 mM α-ketoglutarate). Similar results were obtained with tyrosine transaminase; βMG was found to be an inhibitor with respect to tyrosine. However, 100 mM βMG caused only a 40% inhibition of the rate of conversion of 2 mM L-tyrosine to p-hydroxyphenylpyruvate.

No evidence was obtained that βMGln or βMAsn were substrates or inhibitors of glutamine transaminase L, glutamine transaminase K and asparagine transaminase. Previous work (6-9) has shown that although the substrate specificity of these enzymes is quite broad, α-keto acids and α-amino acids with branching in the β-position do not bind effectively.

When rat brain homogenates were incubated with 10 mM βMA or 10 mM βEA in 100 mM potassium phosphate buffer, pH 7.2, at 25°C for 1 h, the GAD activity declined by 92% and 55%, respectively; βMG, βMGln and βMAsn (10 mM, 2 h incubation) had little effect (<10%) on the GAD activity. βEA has also recently been shown to inhibit cysteinesulfinate decarboxylase (23). Within the limits of sensitivity of the assay (±20%) none of the unsaturated amino acids had any effect on the activity of GABA transaminase in rat brain homogenates (55 mM, 1 h of incubation). These compounds were neither substrates nor inhibitors of D-and L-amino acid oxidases.

Finally, when rat liver homogenates were incubated with 40 mM βMG in 100 mM imidazole-HCl buffer, pH 7.2, for 1 h at 37°C there was no loss of glutamine synthetase activity (assayed by the hydroxamate method of Pamiljans **et al.** (24)). However, βMG appears to be a substrate; when L-glutamate was replaced

with βMG the color yield of hydroxamate was ~10% that obtained with L-glutamate. This observation is in accord with the findings that sheep brain glutamine synthetase can accept methyl substituted glutamates (including the β-methyl isomers) as substrates(25).

DISCUSSION

In view of the ready inactivation of sGAT by βMA (2), it was of interest to prepare and test the corresponding glutamate analogue βMG. Unfortunately, βMG did not inactivate tyrosine and glutamate-alanine transaminases and only slowly inactivated sGAT. βMG was only weakly competitive with the natural amino acid substrates, suggesting that glutamate binds to the active sites of these enzymes without room to spare. In addition, the γ-CH_2 group of βMG isolates the double bond from the electron-withdrawing effect of the side-chain carboxyl group, decreasing the electrophilicity of the double bond. In the case of sGAT it is known that various α- and β-substituted aspartates bind effectively to the active site, e.g. α-methyl- (4, and references cited therein)), **erythro**-β-hydroxy- (26) and α-hydroxymethylaspartate (27). Recent crystallographic data indicate that the methyl group of α-methyl-L-aspartate, bound to crystalline sGAT, protrudes to the surface (4). Jenkins (28) has shown that **erythro**-β-hydroxyaspartate is an effective competitive inhibitor of sGAT with respect to aspartate, but it is a substrate with an extremely low turnover rate (0.02% the rate exhibited by aspartate). In the present work, we found that βEA was also an effective competitive inhibitor with respect to aspartate and a substrate with a comparably poor turnover number. We had hoped that βEA would irreversibly inhibit sGAT but not GAD, but the present work showed that βEA reversibly inhibits sGAT. We had considered the possibility that the inhibition of GAD by βMA and βEA was of the irreversible suicide type; however, the very recent finding that aspartate and β-methylaspartate cause a conformational change in GAD that lowers the V_{max} of GAD for glutamate (29) suggests that alternative mechanisms may be operating. The mechanism of the inhibition of GAD by βMA is currently under investigation. It still may be possible to design a selective inhibitor, based on the aspartate backbone, of the type: $HO_2C(=CHX)CH(CO_2H)NH_2$,

where X is an electron-withdrawing group such as F or $-CO_2H$. With the topology of the active site becoming known from x-ray crystallographic studies (4), it might even be possible to design the molecule in such a way that X fits into a pocket close to the active site.

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