FLUORINATED AMINO ACIDS AND PHOSPHOPYRIDOXYL FLUOROAMINO ACIDS AS REVERSIBLE ACTIVE SITE DIRECTED INHIBITORS OF ASPARTATE TRANSAMINASE[1]

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Trifluoromethionine and p and o-monofluorophenylalanine can be accepted by cytoplasmic aspartate transaminase as substrates; α-trifluoromethyl-DL-alanine can act as an inhibitor of the enzyme. Covalent pyridoxal phosphate (PLP) derivatives of these amino acids can be prepared and after reduction of the resulting Schiff's base with $NaBH_4$ the isolated PLP-fluorinated amino acids used as inhibitors of apoenzyme. The union of the fluorinated-PLP compounds to apoenzyme is stoichiometric as judged by fluorescence and circular dichroism techniques as well as by inhibition of enzymatic transamination with the natural substrates, aspartate and α-ketoglutarate. The transaminase retains its stereospecific discrimination at the substrate level and when presented with a racemic mixture of fluorinated (DL) amino acid-PLP compound accepts only one isomer. The holoenzyme-fluorinated substrate complexes are stable to dialysis and chromatography through Sephadex G-25, yet, they can be resolved by phosphate buffers into native apoenzyme and fluorinated amino acid-PLP compound.

Enzymes that utilize PLP[4] as coenzyme undergo covalent catalysis with participation of the PLP and formation of a Schiff's base between the amino acid and the coenzyme. The trapping of

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[4]Abbreviations: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; GAT, glutamate aspartate transaminase; trifluoromethionine, 2-amino-4-(trifluoromethylthio)butanoic acid.

this substrate-coenzyme-enzyme complex has proved elusive since attempts to reduce the Schiff's base in the complex lead to a preferential reduction of the internal PLP-lysyl aldimine of the holoenzyme. In a related enzyme, the pyridoxamine pyruvate transaminase, the chemically synthesized pyridoxyl-amino acid compounds, with properties of both substrate and coenzyme, bind to the enzyme with higher affinity than either substrate or products of the reaction (1). The pyridoxyl-amino acid derivatives were effective even when using amino acids that are not utilized as substrates by the free enzyme (1). On the other hand, inhibition of apoenzymes of PLP-dependent enzymes by the phophorylated derivatives of pyridoxyl amino acids leads to only partial inhibition even though these enzymes are known to have extremely high affinities ($K_d > 10^{-10}$) for either PLP or PMP (2).

The introduction of fluorinated amino acids at the active site region of enzymes is desirable to widen the use of fluorinated substrates and for the exploitation of the spectroscopic properties of the fluorinated compounds as probes of enzymatic active site environments (3-5). To this purpose the introduction of chemically prepared PLP-fluorinated amino acids can be an excellent beginning. This technique could also, in principle, be used to introduce the fluorine label to the active site region of other enzymes which accept so-called "transition state analogues" (6).

We describe the preparation of the PLP complex with fluorinated amino acids and its highly specific effect on the active site region of GAT.

METHODS AND RESULTS

Synthesis of phosphopyridoxyl trifluoroamino acids

The amino acids (1.2 mmole) α-trifluoromethyl-DL-alanine,

synthesized by the method of Christensen and Oxender (7), trifluoro-L-methionine (Cyclo Chemical Corp.) DL-o-fluorophenylalanine (Pierce Chemical) and DL-p-fluorophenylalanine (Sigma) were dissolved in 10 ml of methanol at 5° and mixed with 1 mmole of neutralized PLP. After 30 minutes the resulting Schiff's base was reduced by the slow addition of 5 mM $NaBH_4$. The solution was brought to pH 5.5 with acetic acid and evaporated to dryness. This material can be dissolved in water and chromatographed on a 2.5 x 50 cm Amberlite CG-50 column and eluted with water. Fractions with absorbance at 330 nm were pooled and further fractionated by descending paper chromatography in Whatman 3MM paper using t-butanol-formic acid-water (15:15:70) as the eluent. The compounds could be detected by their ultraviolet fluorescence and their properties are shown in Table I.

Fluorinated amino acids as substrates of GAT

The fluorinated amino acids, except α-trifluoromethyl-DL-

TABLE I

Properties of phosphopyridoxyl derivatives of fluorinated amino acids

PLP-derivative of		m.p.	absorption max. pH 7.0 nm	ε_{324} [a]
α-trifluoromethyl-DL-alanine	(I)	209 ± 1	324, 255	7000
Trifluoro-L-methionine	(II)	205 ± 1	324, 255	7000
DL-p-fluorophenylalanine	(III)	198 ± 1	324, 255	6900
DL-o-fluorophenylalanine	(IV)	197 ± 1	324, 255	7000

[a] 1 cm light path.

TABLE II

Dissociation and inhibition constant of fluorinated amino acids with GAT

Amino acid	K_d[a]	K_i[b]
	mM	mM
Trifluoro-L-methionine	132	142
α-trifluoromethyl-DL-alanine	29	40
DL-o-fluorophenylalanine	53	68
DL-p-fluorophenylalanine	8.2	15

[a]From spectrophotometric methods (8,9).

[b]As competitive inhibitors of transamination between aspartate and α-ketoglutarate.

alanine, were tested as substrates of GAT by measuring the half times of their direct interaction with the pyridoxal form of the transaminase, absorbance at 360 nm, to convert to the pyridoxamine form (8), absorbance at 330 nm. α-Trifluoromethyl-DL-alanine was looked at as a competitor with α-methylaspartate for formation of an enzyme·α-methylaspartate complex with absorbance at 430 nm (9) Table II. All fluorinated amino acids also act as competitive inhibitors of substrates of the enzymatic transamination between aspartate and α-ketoglutarate.

Phosphopyridoxyl fluoroamino acids as inhibitors of GAT

Compounds I through IV (Table I) can be added to the apoenzyme and the resulting complex is unable to combine with pyridoxal phosphate to either give the well-known absorption spectrum of the holoenzyme or generate catalytic activity (Table III). The combi-

TABLE III

Properties of phosphopyridoxyl fluoroamino acid-apoGAT complexes

1×10^{5} M apoenzyme was incubated for 1 hour at room temperature with compounds I-IV, 2 to 3×10^{-5} M, or PLP, 1×10^{-4} M in 0.05 M Tris-HCl buffer, pH 8.2. The mixture was passed through a 2 x 30 cm column of Sephadex G-25 equilibrated and eluted with the same buffer. Apoenzyme conversion of complexes of compounds I-IV and GAT was carried out by addition of an equal volume of 2 M potassium phosphate buffer, pH 6 at room temperature for 2 hours.

Complex	Absorbance 280/330 nm	Activity %
ApoGAT	43	5
Apoenzyme+PLP	-	100
Apoenzyme+I	8.7	6
Apoenzyme+II	9	5.5
Apoenzyme+III	8.5	7
Apoenzyme+IV	8.5	6.5
Complexes of I-IV and GAT+PLP	-	7.3
Complexes of I-IV and GAT converted to apoenzyme then add PLP	-	98

nation between complexes I-IV and apoenzyme can be achieved even with incubation of stoichiometric amounts (2×10^{-4} M) of each component in a 0.05 M tris-HCl buffer, pH 8.0. Passage through a 2 x 40 cm Sephadex G-25 column or dialysis against distilled water for several days did not resolve the enzyme PLP-fluorinated amino acid complex into apoenzyme and PLP-fluorinated substrate. The

inhibition of catalysis or the formation of the spectroscopically detectable complex produced by the presence of compounds I-IV on the apoenzyme is not an irreversible process. Regeneration of catalytic activity due to the formation of a viable apoenzyme can be achieved by adjustment of the fluorinated compound (I to IV) - enzyme complex with potassium phosphate buffer, pH 6.0. Under these conditions there is liberation of the fluorinated compound (I-IV) and regeneration of apoenzyme which after the subsequent addition of PLP or PMP is catalytically indistinguishable from native holoenzyme.

Spectral properties of the enzyme•substrate-analogue complex

Pyridoxamine phosphate, if excited at 330 nm, fluoresces in the 400 nm region; binding to the apoenzyme results in a substantial quenching of this fluorescence (10). Compounds I-IV exhibit similar properties. Figure 1 shows the varying degree of quenching observed in a titration of the active site of the apoenzyme with one of the fluorinated complexes. Complete quenching can be

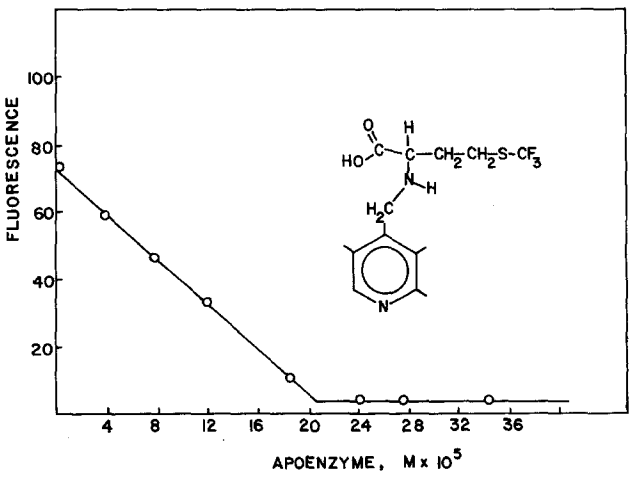

Figure 1. Fluorescence quenching of 2.1×10^{-6} M trifluoromethylmethionine-PLP in 0.1 M Tris-HCl buffer, pH 8.1 upon addition of increasing aliquots of apoenzyme.

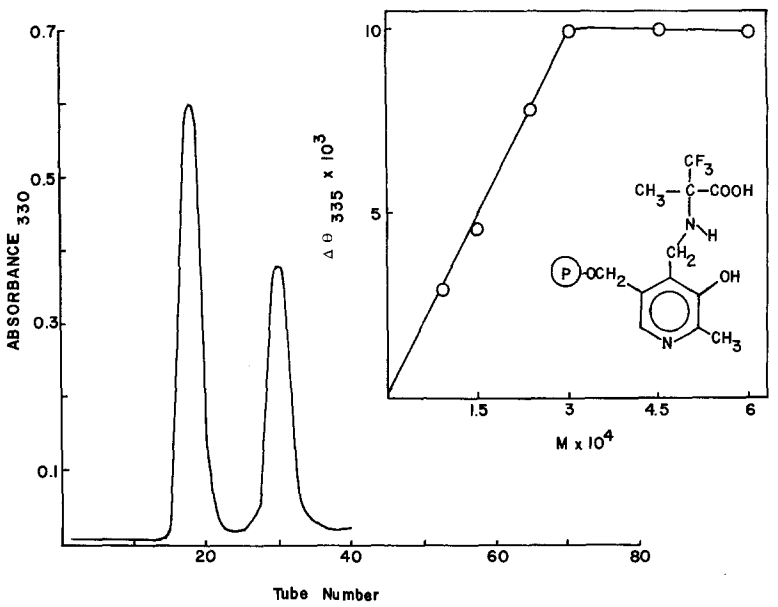

Figure 2. Elution profile of a mixture of 1.2×10^{-4} M of compound I and 1.2×10^{-4} M apoenzyme through a 2 x 40 cm Sephadex G-25 column equilibrated and eluted with 0.1 M Tris-HCl buffer, pH 8.1, first peak protein, second peak free compound. Insert, positive circular dichroism induced by binding of compound I to 1.5×10^{-4} M apoenzyme in the same buffer. Similar results were obtained with racemic compounds III and IV.

observed with compound II but a residual fluorescence remains in the bound compounds (I, III and IV). Two equivalents of racemic compounds I, III or IV are needed to achieve saturation of the apoenzyme. If one equivalent of the racemic compounds is added only half combines with the apoenzyme and half the fluorescence remains. This mixture can be fractionated through a Sephadex G-25 column resulting in the elution profile of Figure 2. The first peak also contains the ultraviolet absorbance of the protein and half the specific activity of native apoenzyme. The absorbance ratio, 280 nm/330 nm, of this peak corresponds to that of a transaminase with half the active sites occupied by the fluorinated compound. Similar treatment with only the L isomer of trifluoromethionine (compound II) produces one elution peak in the protein

region void of enzymatic activity. Thus, it appears that when presented with a racemic compound the enzyme accepts only one isomer.

A different approach to determine binding of the analogues of PLP to the active site is the formation of a dichroic signal at the absorption maximum of compounds I-IV. The dichroic bands are due to the chiral environment provided by the apoenzyme to the chromophore under observation and the increase in chirality is proportional to the amount of PLP derivative bound at the active site (11). Titrations of the active site by this technique also results in an stoichiometric binding of 1 mole of one isomer per active site (Insert Fig. 2).

DISCUSSION

The fluorinated amino acids used in this work are acceptable substrates for cytoplasmic GAT and α-trifluoromethyl alanine is an inhibitor of the enzyme because of competition with amino acids for the active site. Once coupled to PLP as phosphopyridoxyl fluoro amino acid, the binding to the active site is stoichiometric. This union can be accomplished even at equimolecular concentrations of compound (I-IV) to apoenzyme; this observation and the stability of enzyme·coenzyme-substrate complex to passage through Sephadex G-25 column or dialysis indicates a great affinity of the apoenzyme for compounds I-IV. Indeed, not even the coenzyme, PLP or PMP, or a combination of amino acid and coenzyme can displace compounds I-IV from the active site region and regain catalytic activity. This is an unexpected result since only partial inactivation has been reported with the PLP derivatives of standard (protonated) amino acid substrates even when used at concentrations as high as 10^{-3} M (2). Part of this discrepancy, however, can now be ascribed to the presence of phosphate buffer

and the pH used in studies of the combination of the apoenzyme to the protonated complexes (2). The inhibition obtained with PLP-fluoroderivatives is what can be expected if the active site accepts both the PLP and substrate moieties, a strong binding that can hardly be displaced with excess PLP.

Since regeneration of apoenzyme can be achieved by removal of the compounds I-IV by mild methods, denaturation or covalent bond formation between complexes I-IV and apoenzyme seems to be absent in the formation of the coenzyme-substrate·apoenzyme complexes. This behavior contrasts with that of phosphopyridoxyl glutamate which inactivates GAT by forming an acid stable covalent bond with a threonyl residue in the protein (12).

True enzyme substrate complexes of GAT can produce positive, negative or no dichroic bands in the region of PLP absorbance. Compounds I, III and IV produce positive dichroicity at 330 nm in the presence of the apoenzyme and compound II does not produce dichroicity at this wavelength. These observations, then, are not in disaccord with a general proposal of binding of all compounds to the active site region. This concept is further strengthened by the fluorescent quenching of all four compounds when bound to apoenzyme and the stoichiometric inactivation of enzyme catalysis.

It is of interest that the enzyme retains stereospecificity with respect to the substrate isomer it can accept at the active site. In all cases where it is presented with DL racemic mixture of the fluorinated amino acid-PLP complex, it binds only half the corresponding amount of the racemic compound. This observation also indicates that the enzyme not only binds compounds I-IV through the pyridine and phosphate ligands provided by the PLP moiety but also provides ligands and/or stereospecific arrange-

ment able to accommodate only one substrate isomer. That is, even in the forced acceptance of substrates through multiliganded binding to the subsites of the active center, a strict stereoselectivity is maintained by apotransaminase.

It is concluded that the fluorinated amino acids can successfully be introduced at the active site, reasserting the excellence of GAT as an enzyme of choice to further explore the spectroscopic properties of stoichiometrically bound fluorinated substrates in the active site region of enzymes.

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