Inhibition of L-Glutamic Acid Decarboxylase by Cycloglutamates*

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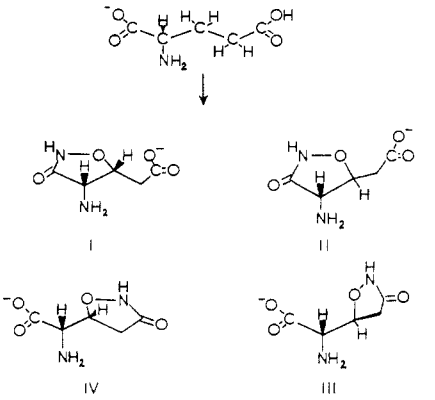
ABSTRACT: Four isomeric cyclic derivatives of DL-glutamic acid, *threo*-α-cycloglutamate (I), *erythro*-α-cycloglutamate (II), *erythro*-γ-cycloglutamate (III), and *threo*-γ-cycloglutamate (IV) have been tested as inhibitors of L-glutamate decarboxylase of *Escherichia coli*. In short-time tests all act as competitive inhibitors; the threo compounds (I and IV) have K_I values of 1.1–1.3 × 10^{-3} M at pH 4.8 and are suggested to possess conformations similar to those of the substrate. The erythro compounds II and III are bound more tightly with K_I values of 9 × 10^{-5} and 1.8 × 10^{-5} M, respectively. All of the EI complexes undergo slow changes in spectra. Compound III causes only a small initial change in the enzyme spectrum, but over a period of about 3 hr the absorption band shifts from 420 to 380 nm with loss of circular dichroism. (Oximes of the enzyme, by contrast, possess easily measurable dichroism.) Activity can be almost fully restored by substrate at this stage. Reduction of the EI complex with sodium borohydride yields principally *N*-pyridoxyl-III. The 380-nm band is suggested to represent a Schiff's base in which the C=N bond is not coplanar with the pyridine ring, rotation into the planar position being blocked by the rigid conformation of the inhibitor. On longer standing inhibition becomes irreversible. Gel filtration, with or without borohydride reduction yields the oxime of pyridoxal with *erythro*-β-aminooxyglutamate. Thus the protein has catalyzed ring opening in the inhibitor. Curiously, only dephosphorylated derivatives of the coenzyme are obtained upon gel filtration and reduction with borohydride also leads to dephosphorylation. Reduction of the ES complex with glutamate produces *N*-pyridoxylpyroglutamic acid, suggesting that the γ-carboxyl group of glutamate lies close to the nitrogen of the enzyme–substrate Schiff's base and that reduction is accompanied by ring closure.

The creation of highly effective inhibitors offers one means of regulating enzymic processes. L-Glutamic acid decarboxylase (GAD),[1] one of the enzymes which controls the level of γ-aminobutyric acid in tissues of the central nervous system, is inhibited by a wide variety of substances, the most active of which are derivatives of hydroxylamine (Roberts and Frankel, 1951; Della Pietra *et al.*, 1963; Saschenko *et al.*, 1968). These hydroxylamines are not specific but inhibit many different PLP-dependent enzymes by formation of oximes with the coenzyme.

A new class of specific inhibitors which are cyclic analogs of amino acid substrates and which also possess acylating properties has been designed. The carboxyl group of the amino acid (*e.g.*, alanine, phenylalanine, tyrosine, and glutamic acid) has been substituted by a cyclic hydroxamic ether group (Khomutov *et al.*, 1965–1968).

Four isomeric compounds of this type were prepared as analogs of glutamic acid (Khomutov *et al.*, 1965). In two of the compounds, the *threo*-α- and *erythro*-α-cycloglutamates (*cis*- and *trans*-5-carboxymethyl-4-aminoisoxazolidone-3, I and II), the α-carboxyl group participates in formation of the heterocyclic system while in the other two, *erythro*- and *threo*-γ-cycloglutamates (*erythro*- and *threo*-5-carboxyaminomethylisoxazolidone-3, III and IV), the γ-carboxyl group is used in forming the ring. In these substances all of the basic elements of the substrate which might permit strong binding of inhibitor to the substrate site of an enzyme are conserved



and in a rigidly fixed conformation. Thus, for example, a proton from the isoxazolidone group dissociates with a pK_a of about 4.4 (Neilands, 1956), mimicking the corresponding carboxylate group in the substrate. In addition, the acylating property of the substances provides the possibility of blocking functional groups of the enzyme essential for catalysis.

It has been shown previously that compound I has a very high affinity for aspartate aminotransferase of pig heart and that both I and III react in specific but distinctly different ways with that enzyme (Khomutov *et al.*, 1967a, 1968). Compound I is also an extremely good inhibitor of the transaminase of γ-aminobutyric acid (Severin *et al.*, 1968). The present work describes the interaction of the same compounds with GAD from *Escherichia coli*.

Materials and Methods

Compounds. The four isomeric cycloglutamic acids (I–IV) were obtained according to Khomutov *et al.* (1965) and were recrystallized from water. The purity was verified by electro-

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[1] Abbreviations used are: GAD, L-glutamate decarboxylase; PLP, pyridoxal phosphate; EI and ES, enzyme–inhibitor and enzyme–substrate complexes; BAP, β-aminooxypropionic acid; BAGA, *erythro*-β-aminooxyglutamic acid; *A*, absorbance.

phoresis and also by the absence of absorption at 380 nm after mixing with soluble aspartate aminotransferase of pig heart. *erythro*-β-Aminooxyglutamic acid (BAGA) was obtained by hydrolysis of II or III by concentrated HCl at 37° and purification by preparative electrophoresis.

Oximes of pyridoxal with BAGA and with β-aminooxypropionic acid (BAP) were obtained in alcoholic solutions from pyridoxal and the corresponding hydroxylamines. The *N*-pyridoxyl derivative of III was obtained by reduction with sodium borohydride of the Schiff base of pyridoxal with III (Severin *et al.*, 1969). *N*-Pyridoxylpyrrolidonecarboxylic acid was obtained by treatment of *N*-pyridoxyl-(5-phosphate)-pyrrolidonecarboxylic acid with a 1% solution of alkaline phosphatase at pH 8.2.

L-*Glutamate decarboxylase* (EC 4.1.1.15) was extracted from harvested cells of *E. coli var. communior* and was purified 200-fold using a modification of the method of Shukuya and Schwert (1960) to a specific activity corresponding to about 60% homogeneity. The specific activity was from 18,000 to 20,000 units (μl of CO_2 evolved in 10 min at 37° per mg of protein). Activity was determined in the Warburg apparatus as described by Saschenko *et al.* (1968). The final concentration of the substrate, L-glutamic acid, was 0.01 M; the pH of 4.6 was maintained with 0.1 M pyridine-HCl buffer. Protein was determined by the method of Lowry *et al.* (1951) or by absorption at 280 nm.

Ultraviolet spectra were measured with the Hitachi Model EPS-2 recording spectrophotometer and circular dichroism spectra with the Jouan dichrograph.

Electrophoretic separations were carried out on Whatman No. 1MM paper with the following solvent systems: pyridine–acetic acid–water (1:10:189, v/v, pH 3.5); pyridine–acetic acid–water (100:3:900, v/v pH 6.5); 100 V/cm, 10–20 min. Electropherograms were sprayed with ninhydrin, with 2,6-dichloroquinonechlorimide or sodium nitroprusside and were examined under uv light.

Determination of the Degree of Inhibition. Into the main compartment of each of a series of Warburg vessels were placed 1.6 ml of 0.1 M pyridine-HCl buffer (pH 4.6), 0.2 ml of inhibitor of appropriate concentration, and 20 μl of enzyme solution (0.005 mg of protein). After 10-min incubation at 37°, 0.4 ml of 0.05 M L-glutamic acid was added and the activity of the enzyme was determined. Several concentrations of inhibitor were used and the rates were compared with that in the absence of inhibitor and were used to estimate $[I]_{50}$, the inhibitor concentration giving 50% inhibition. The value of $[I]_{50}$ together with the Michaelis constant for L-glutamic acid was used to estimate the values of the inhibition constant, K_I, for competitive inhibition given in Table I.

Standard Procedure for Studies of the Reaction of Cycloglutamates (I–IV) with GAD. A fresh 1.0×10^{-2} M solution of cycloglutamate was prepared by dissolving 1.44 mg of the crystalline inhibitor in 0.9 ml of 0.2 M buffer. A 0.7-ml portion of this solution was mixed with 0.3–0.4 ml of enzyme and 1.0–0.9 ml of 0.2 M buffer. The final volume was 2.0 ml, the final enzyme concentration was 3.5 mg/ml, and the inhibitor concentration 3.5×10^{-3} M. The term, EI, followed by (in parentheses) the time of incubation, will denote the inhibited forms of the enzyme in such a reaction mixture at various stages of inhibition.

Spectrophotometric Study of Inhibition. The effect of the cycloglutamate inhibitors on the spectrum of GAD was observed in 0.2 M acetate buffer (pH 4.7) and in 0.2 M phosphate buffer (pH 6.0) at 20°. The spectrum of the solution was recorded at various times over a 24-hr period and samples were taken periodically from the same solution for determination of enzymatic activity. Solutions for measurement of the circular dichroism spectrum were prepared in the same way.

TABLE I: Inhibition of Glutamate Decarboxylase by Cycloglutamates.

	Compound	Inhibn Constant (M)[a]
I	*threo*-α-Cycloglutamate	1.3×10^{-3}
II	*erythro*-α-Cycloglutamate	9×10^{-5}
III	*erythro*-γ-Cycloglutamate	1.8×10^{-5}
IV	*threo*-γ-Cycloglutamate	1.1×10^{-3}

[a] Under similar conditions, the Michaelis constant for L-glutamate was 2.2×10^{-3} M.

Gel Filtration of the EI Complex. A solution of EI (24 hr), with maximum absorption at 380 nm, was passed through a column of Sephadex G-25 (2 × 35 cm) which had been equilibrated with water or with 0.05 M Tris-HCl buffer (pH 7.2). Fractions of volume 4.7 ml were collected at a flow rate of 2.35 ml/min per cm^2; those containing not less than 0.5 mg/ml of protein were pooled and spectra were measured. The low molecular weight fraction from the column was also collected and desalted as follows. The pH was adjusted with 1 N sodium hydroxide to 8.5 and the solution was passed through a column (1.8 × 6 cm) of Dowex 1-X2, 50–100 mesh in the acetate form. The column was washed with 50 ml of water and the derivative of vitamin B_6 remaining on the column was eluted with 20% acetic acid at a flow rate of 2.35 ml/min per cm^2. The pooled fractions (4.7 ml each) containing the derivative of vitamin B_6 were combined, evaporated, and investigated electrophoretically.

Reduction of EI and ES Complexes with Sodium Borohydride. A solution (2.0 ml) of the appropriate complex, containing 3.5 mg/ml of enzyme, was cooled to 0° and 0.1 ml of a freshly prepared 1 M solution of sodium borohydride was added. Spectra were recorded after 10 min and the same samples were then subjected to gel filtration and electrophoresis as described in the preceding paragraph.

Determination of the Extent of Decarboxylation of III. The measurements were made exactly as described for determination of activity except that after 10-min incubation of the enzyme with buffer in the Warburg apparatus 25 μmoles of III in 1 ml of 0.1 M pyridine buffer (pH 4.6) was added from the side arm instead of the usual 15 μmoles of glutamic acid. Evolved CO_2 was measured at 10-min intervals for 40 min.

Reactivation of the EI Complex by Substrate. During the inhibition reaction aliquots, each containing 0.005 mg of protein, were removed from the solution of EI complex. To each aliquot, in the main compartment of a Warburg flask, was added 1.8 ml of 0.1 M pyridine-HCl buffer (pH 4.6) and after 2-min incubation at 37° 0.4 ml of 0.05 M L-glutamic acid in 0.1 M acetate buffer (pH 4.6) was added from the side arm. Evolved CO_2 was measured after 5, 10, and 15 min and the velocity of the enzymic reaction was compared in the presence and absence of inhibitor to determine the per cent inhibition.

Possible reactivation of the EI complex by addition of PLP was sought by making measurements exactly as for reactivation by substrate except that in place of 1.8 ml of

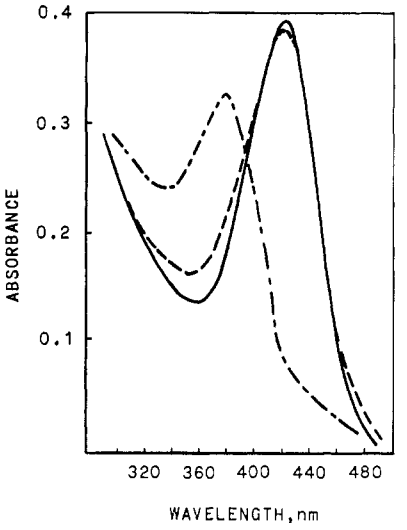


FIGURE 1: Ultraviolet spectra of GAD (3.5 mg/ml) in the presence of 3.5×10^{-3} M *erythro*-γ-cycloglutamate (III); (—) GAD in 0.2 M acetate buffer, pH 4.8; (- - - - -) GAD + III, 1 min after mixing (—·—·) GAD + III, 24 hr after mixing.

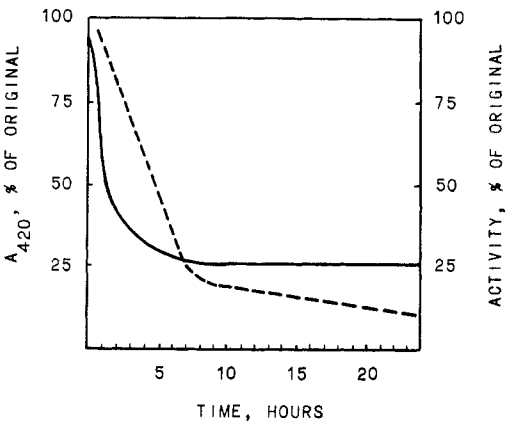


FIGURE 2: Reactivation of the EI complex (3.5 mg/ml of GAD, 3.5×10^{-3} M inhibitor) by an excess of substrate: (—) the disappearance of the absorption at the 420-nm maximum with time; (- - - - -) activity of the enzyme after dilution of a small portion with buffer and addition of glutamate.

buffer, the main compartment of the Warburg flask contained 1.75 ml of the buffer plus 0.05 ml of 4 mM PLP.

Results

The effects of all four isomeric cycloglutamates (I–IV) on the activity of GAD were compared using a 10-min activity assay. Table I gives the apparent inhibition constants, assuming reversible inhibition. The *erythro*-γ-cycloglutamate, cyclized through the γ-carboxyl group (III), was the most active with $K_I = 1.8 \times 10^{-5}$ M. Most of the remaining studies were made exclusively with this isomer, but it should be noted that the *erythro*-α-cycloglutamate (II), cyclized through the α-carboxyl, is also a strong competitive inhibitor and that the other two isomers, although much less active, show values of K_I very similar to the K_M for L-glutamate.

Since III, like the substrate, has both a free carboxyl group and a free amino group, it might be expected to combine with the coenzyme and to undergo decarboxylation. However, upon long incubation (40 min) of the enzyme with an excess of III no decarboxylation was observed.

In further experiments inhibitor was added to highly purified enzyme and changes in the ultraviolet spectrum were recorded at various times. Simultaneously the activity and the degree of reactivation by both substrate and coenzyme as well as the induced optical activity (circular dichroism, CD) were measured. Finally, the solution of EI complex was subjected to gel filtration and both the low molecular weight and protein fractions were investigated. Similarly, the EI complex after reduction with sodium borohydride at various stages of reaction was studied and the results were compared with those obtained in parallel experiments with the corresponding complexes of GAD with L-glutamate, with β-aminooxypropionic acid (BAP), and with β-aminooxyglutamic acid (BAGA).

Interaction of the enzyme with an excess of III at pH 4.8 in 0.2 M pyridine-HCl or 0.2 M acetate buffers led to a small initial change in spectrum followed by a slow, major change. During the first few seconds after addition of the inhibitor the absorption at 420 nm decreased slightly while the absorption at 340 nm increased by a similar amount (Figure 1, 1 min). These spectral changes completely coincide with those observed upon addition of the substrate, L-glutamate (see Figures 5 and 4 of Shukuya and Schwert, 1960). However, in the case of the cycloglutamate the initial enzyme spectrum is not restored (as it is with substrate) because the inhibitor molecule is not broken down by the enzyme.

Following the initial rapid change, the characteristic 420-nm absorption of the enzyme slowly disappeared and a new absorption band was formed at 380 nm (Figure 1, 24 hr). Further incubation, for another 24 hr, led to no further change in spectrum. Similar, but slower changes were seen with compounds I, II, and IV.

From the spectral studies and the behavior of III as a competitive inhibitor in 10-min assays, two stages of inhibition can be recognized: the first causing only a minor change in spectrum and the second a shift of the absorption peak to 380 nm. A third stage of inhibition was established by study of the reactivation of the EI complex with an excess of substrate. For example, after 2 hr the change in spectrum was almost complete, but the enzyme could be reactivated by substrate to over 80% of the original activity (Figure 2). Thus the second stage of inhibition is reversible. However, after 8 hr very little activity was regained in the presence of substrate, which is evidence for a third, irreversible step in the inhibition reaction.

Addition of PLP to the EI complex does not restore activity to the enzyme at any stage. The EI complex with absorption maximum at 380 nm possesses no CD either in the second or third stage of inhibition as shown by the complete disappearance of the positive CD band of the enzyme at 420 nm ($\Delta A/A = 1.8 \times 10^{-3}$). When enzyme is incubated with an excess of substrate under the same conditions the spectrum changes only slightly but the CD disappears completely and then returns upon exhaustion of substrate. This loss of CD in the ES complex has been reported in more detail by Huntley (1970), who incubated GAD ($A_{420} = 0.3$) with 0.01 M glutamate at pH 4.6, 0°. Immediately after addition of substrate the absorbance at 420 nm fell by about 15% and the CD, measured 1–1.5 min after mixing fell to less than 10% of its initial value. Both absorbance and CD had returned to their original values within 3 min as the substrate was exhausted.

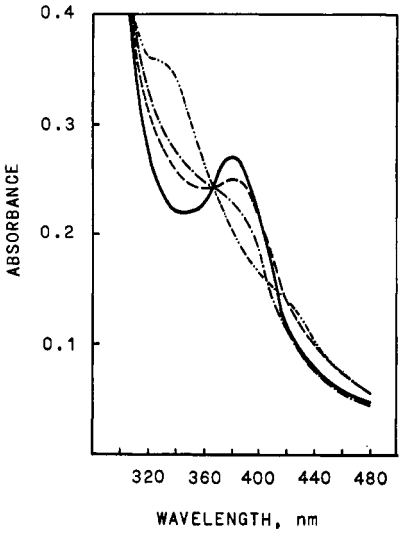


FIGURE 3: Changes in the uv spectra of GAD in the presence of III following changes in pH; (—) GAD + III incubated at pH 4.8; (- - - -) same solution after change of pH to 5.8; (—·—·—) GAD + III incubated at pH 6.15 (- -·- -); same solution after change of pH to 6.55. The concentration of GAD was 3.5 mg/ml and that of the inhibitor was 3.5×10^{-3} M.

The effect of pH on the spectral changes was investigated by observing the reaction at both pH 4.8 and at pH 6.15 for 24 hr and then changing the pH to the higher values of 5.8 and 6.65, respectively, with the results shown in Figure 3. The final effect of the increase in pH (from pH 4.8) was almost independent of whether the change was made prior to incubation with inhibitor or after 24 hr. The characteristic EI absorption band at 380 nm decreased with increasing pH and inflections appeared at about 330 and 430 nm. At pH 6.3 (0.25 M phosphate buffer), the enzyme and inhibitor reacted very slowly, over a 24-hr period.

When EI (24 hr) was subjected to gel filtration through Sephadex G-25 (equilibrated with water or with 0.05 M Tris-HCl buffer, pH 7.2), the coenzyme, in combination with the inhibitor, was split off and completely separated from the protein fraction. The activity of the apoenzyme so obtained was not restored by incubation with PLP whereas gel filtration, under identical conditions, of enzyme which had been resolved by treatment with α-methylglutamate (Huntley and Metzler, 1968; O'Leary, 1969) gave apoenzyme which was restored to 60% of the original activity by PLP. The low molecular weight fraction from gel filtration was desalted and studied by high-voltage electrophoresis at pH 3.5. Two basic spots were observed on the electrophoregrams, a major spot corresponding in electrophoretic mobility to pyridoxal and a minor one to the oxime of pyridoxal with BAGA (Figure 4A).

Reduction of EI (5 hr) by $NaBH_4$ led to a decrease in absorption at 380 nm and an increased absorption at 330 nm (Figure 5). Gel filtration of the reduced enzyme yielded inactive apoenzyme. Electrophoresis of the low molecular weight fraction revealed two spots corresponding in mobility to the oxime of pyridoxal with BAGA and to *N*-pyridoxyl-III (Figure 4B). The EI (5 hr), which could be reactivated 50% by substrate yielded more *N*-pyridoxyl-III than oxime. On the other hand, EI (24 hr), for which there is practically no reactivation by substrate, yielded predominately the oxime and little pyridoxyl-III. Thus, stages 2 and 3 of the inhibition can be distinguished chemically very clearly.

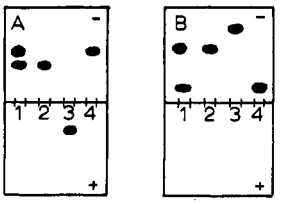


FIGURE 4: Electrophoregrams of low molecular weight fraction after separation by gel filtration. (A) From the EI complex (24 hr); electrophoresis at pH 3.5, 10 min, 80 V/cm: (1) reaction mixture; (2) oxime of pyridoxal with BAGA; (3) pyridoxal phosphate; (4) pyridoxal. (B) From borohydride-reduced EI complex. Electrophoresis at pH 3.5, 10 min, 80 V/cm; (1) reaction mixture; (2) oxime of pyridoxal with BAGA; (3) pyridoxal; (4) pyridoxyl-III.

The O-substituted hydroxylamines, BAP and BAGA, react with the enzyme to yield oximes, also with appearance of an absorption maximum at 380 nm (Figure 6). In the case of BAP the oxime was fully formed 1 min after mixing; but BAGA reacted slowly over a period of 4 hr. Addition of an excess of substrate immediately after formation of the oxime did not reactivate the enzyme. Both EI complexes possessed optical activity in the absorption band. (The oxime of the enzyme with β-aminooxypropionic acid possessed a positive CD [$\Delta A/A = 0.67 \times 10^{-3}$], that with β-aminooxyglutamic acid a negative CD [$\Delta A/A = 0.18 \times 10^{-4}$].) Gel filtration of the EI complex yielded inactive apoenzyme and, in the low molecular weight fraction, the oximes of pyridoxal with BAP and BAGA, respectively.

Reduction by $NaBH_4$ of the ES complex at pH 4.6 led to disappearance of the absorption maximum at 420 nm, the appearance of absorption in the 330-nm region (Figure 5), and complete loss of activity of the enzyme. Upon gel filtration of the reduced ES complex a partial dissociation (>30%) of the coenzyme, in combination with glutamic acid, was observed. Electrophoretic investigation revealed a spot which was identified as a product derived from *N*-pyridoxyl-glutamic acid, namely, its lactam, *N*-pyridoxylpyroglutamic acid (Figure 7).

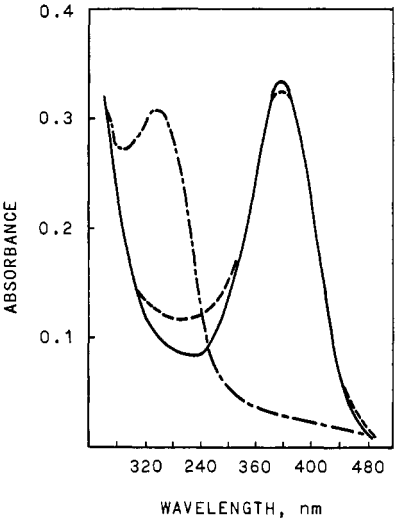


FIGURE 5: Uv spectrum of ES complex (3.5 mg/ml of GAD and 1×10^{-2} M L-glutamate) after reduction with $NaBH_4$: (—) GAD in 0.2 M acetate buffer (pH 4.8). (- - - -) GAD + substrate 1 min after mixing; (—·—·—) ES complex 10 min after addition of $NaBH_4$.

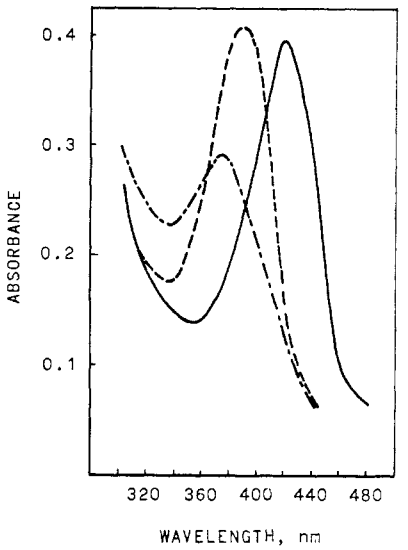


FIGURE 6: Uv spectra of GAD in the presence of O-substituted hydroxylamines: (—) GAD in 0.2 M acetate buffer, pH 4.8; (- - -) GAD + BAP 1 min after mixing; (—·—) GAD + BAGA 4 hr after mixing.

Discussion

Our results suggest that cycloglutamates, such as III react with glutamate decarboxylase (GAD) in at least three stages as follows (positions of absorption bands are shown in parentheses):

$$\underset{(420\text{ nm})}{E} + I \underset{\text{stage 1}}{\overset{K_I}{\rightleftharpoons}} \underset{\substack{(420\text{ nm})\\ \text{and}\\ 340\text{ nm})}}{EI(1)} \overset{\text{stage 2}}{\underset{\text{slow}}{\rightleftharpoons}}$$

$$\underset{(380\text{ nm})}{EI(2)} \xrightarrow[\text{irreversible}]{\text{stage 3}} \underset{(380\text{ nm})}{EI(3)}$$

The first stage is recognized by the strong competitive inhibition during 10-min activity assays. At saturating inhibitor concentrations the spectrum changes little; the 420-nm peak falls slightly and a small increase is observed at 340–370 nm.

The 420-nm absorption in stage 1 could arise in either of two ways: (1) from the internal Schiff's base originally present in the enzyme and still present in the first EI complex, EI (1). (2) from a Schiff's base of the inhibitor with the bound coenzyme. We prefer the first explanation which accounts better for the subsequent slow steps and for the lack of decarboxylation of the inhibitor.

Cycloglutamates, I–IV, are substrate analogs of rigidly fixed conformation whose action as competitive inhibitors may provide steric information about the substrate binding site. For example, the threo pair, I and IV, possess K_I values similar to the Michaelis constant of the substrate. This observation suggests that the nearly identical conformations drawn for I and IV, in which both the α-NH_2 group and the γ-COOH group are directed to the same side of the ring, may be close to that of the substrate in the Michaelis complex. However, the erythro pair, II and III, in which the α-NH_2 and γ-COOH groups are directed to opposite sides of the ring (see drawings) are even more tightly bound. The K_I for III is about 60 times less than K_M for glutamate and 16 times less than K_I for simple dicarboxylic acid inhibitors such as glutarate ($K_I = 7 \times 10^{-4}$).[2] These results suggest that the structure of compound III is the most complementary to that of the "substrate pocket" and that the increased binding affinity is a result of the rigidly fixed structure imparted by the isoxazolidone ring.

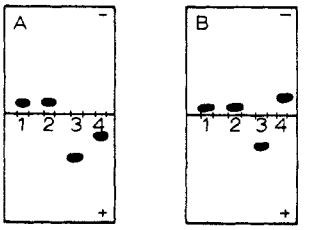


FIGURE 7: Electrophoregrams of the low molecular weight fraction after separation from the $NaBH_4$-reduced ES complex. (A) pH 6.5, 20 min, 80 V/cm; (B) pH 3.5, 10 min, 80 V/cm; (1) reaction mixture; (2) lactam of pyridoxylglutamic acid; (3) pyridoxyl 5-phosphate glutamic acid; (4) pyridoxylglutamic acid.

The small increase in absorption at 340–370 nm during stage 1 of inhibition by III resembles that seen with substrate and may be caused by formation of a small amount of an adduct of the amino group of the inhibitor with the internal Schiff's base of the enzyme. When α-methylglutamate reacts with the same enzyme, such an adduct forms rapidly in large amounts as a transient intermediate during formation of the Schiff's base of this pseudosubstrate with the coenzyme (Huntley and Metzler, 1968). We assume that with III, a small amount of the adduct is in equilibrium with the Michaelis complex. On the other hand, with the true substrate, the adduct is both rapidly formed and rapidly decomposed to yield products so that it appears in a low steady-state concentration.

The second stage is accompanied by formation of a peak at 380 nm which is devoid of optical activity (CD). Since hydroxylamine and substituted hydroxylamines react with GAD to form 380-nm bands, it would be plausible to assume that the cycloglutamate is opened to create a β-aminooxy group which could then displace the amino group (either the ϵ-amino group of the protein or the α-amino group of the inhibitor) from the existing Schiff's base to form a substituted oxime. To test this possibility the substituted hydroxylamines, β-aminooxypropionic acid (BAP) and erythro-β-aminooxyglutamic acid (BAGA) were tested with the enzyme and were observed to yield 380-nm peaks *but with easily measurable CD.* Furthermore, BAGA reacted very slowly (4 hr) suggesting that it was bound initially in the same way as the cycloglutamates, but that the β-aminooxy group (because of its distance from the imine group in the extended conformation in which it is bound) only very slowly reacts to form the oxime of pyridoxal phosphate (this reaction may require partial or complete dissociation of the bound BAGA and reentry with a different orientation). Final proof that the 380-nm-absorbing form in stage 2 is not an oxime was obtained by borohydride reduction as described below.

An alternative explanation for the 380-nm peak formed with III is that it represents an inhibitor–coenzyme Schiff's base but that the imine double bond is not coplanar with the pyridine ring, accounting for the strong shift of the absorption band from 420 to 380 nm. (Like the usual ES complex,

[2] M. L. Fonda, private communication.

this 380-nm complex has no measurable CD.) Direct evidence in support of this proposal was obtained by observation of *N*-pyridoxyl-III as a final product of inhibition after reduction of EI (5 hr) with $NaBH_4$.

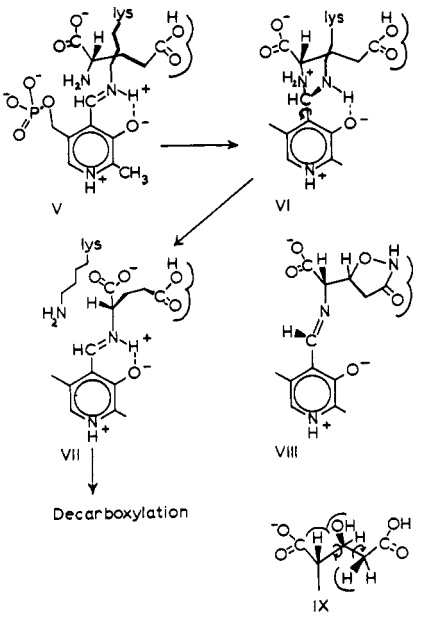
The presence in the inhibitor molecule of the basic elements of the substrate, the high affinity for the enzyme at the binding stage, and the formation of an aldimine with the coenzyme all suggest that the inhibitor might undergo decarboxylation similar to that of the substrate. However, no decarboxylation was observed upon lengthy incubation (40 min) of GAD with an excess of III. Furthermore, only undecarboxylated pyridoxyl-III was obtained as a product of borohydride reduction of EI (5 hr). These facts can be understood if a change of conformation of the protein or of the substrate, or both, is required for decarboxylation.

In agreement with this assumption is the isolation of *N*-pyridoxylpyroglutamic acid after saturation of GAD with glutamate and reduction with sodium borohydride. This suggests that borohydride has reduced the ES complex (Schiff's base) which contains a double bond between substrate and coenzyme but which has not yet undergone decarboxylation. With glutamate this coenzyme–substrate Schiff's base is present at a high steady-state concentration as indicated by the almost complete transient loss of CD in the 420-nm band when acting on glutamate. Furthermore, the formation of a pyroglutamic acid derivative upon reduction by borohydride suggests that in this Schiff's base the γ-COOH group of the substrate must be close to the nitrogen atom of the imine. However, on the basis of the high affinity of the inhibitor, III, we postulate that in the Michaelis complex (V, Scheme I) and in the adduct, VI (340-nm absorption), the γ-COOH group is quite far from the α-amino group.

A simple explanation is that conversion of adduct, VI, to the substrate Schiff's base (VII) requires a significant change in conformation of the substrate or protein or both. We propose that this conformation change brings the carboxyl group closer to the imine nitrogen, *e.g.*, as shown in VII, permitting lactam formation to occur upon reduction with borohydride. (We think it unlikely that the conformation change occurs *during* the borohydride reduction.) While the orientation of the substrate with respect to the coenzyme in the Michaelis complex is unknown, that shown in V, in which the γ-COOH group of the substrate lies on the same side as the 3-OH group of the coenzyme and the α-amino group approaches the imine double bond from behind (to yield adduct, VI with the "S" configuration at the new asymmetric center) is attractive, but other possibilities cannot be excluded. If it is assumed that the γ-carboxyl group of the substrate is firmly anchored at a specific binding site, the conformation change required to convert VI into VII would lead to a "folding" of the substrate into a conformation in which the γ-COOH is closer to nitrogen, as shown in the drawing. The conformation change would also bring the carboxyl group of the substrate into an orientation perpendicular to the plane of the pyridine ring as required for decarboxylation by the hypothesis of Dunathan (1966).

The mechanism of lactam formation during sodium borohydride reduction is not clear but it is of interest that *N*-pyridoxyl 5′-phosphate glutamic acid forms a lactam very rapidly at pH 5 whereas *N*-pyridoxylglutamic acid, under the same conditions, does not cyclize. This suggests that the 5′-phosphate of the coenzyme participates in the cyclization, either alone or in concert with functional groups of the protein. (Note that in the conformation shown in VII a phosphate oxygen could easily contact a proton on the nitrogen of the reduced Schiff's base. On the other hand, if the γ-carboxyl group lies to the opposite side, *i.e.*, toward the phosphate, the phosphate could assist in protonating the γ-carboxyl group.)

SCHEME I



Inhibitors, I–IV, and the substrate assume closely similar conformations at the stage of the initial interaction with GAD and in the reversible formation of the adduct absorbing at 340 nm. The basic difference is observed at the stage of formation of the Schiff's base with the enzyme. The special characteristics of the inhibition may be explained on the basis that I–IV cannot undergo the "folding" necessary to produce the substrate conformation in the Schiff's base, VII. Hence they act as competitive inhibitors in the reversible stages. Because folding is blocked in the inhibitors, the elimination of the ϵ-amino group to form the E–I Schiff's base is slow and leads to a Schiff's base in which the imine group is not coplanar with the pyridine (structure VIII); hence the unexpected position of the absorption maximum at 380 nm and the lack of decarboxylation.

While L-*threo*-β-hydroxyglutamate is decarboxylated by GAD the L-*erythro* isomer is attacked much more slowly (Umbreit and Heneage, 1953; Kaneko and Yoshida, 1962), possibly as a result of hindered rotation during the "folding" of the substrate. As indicated in IX the hydroxyl will be difficult to rotate past both the α-hydrogen and one of the γ-hydrogens of the substrate.

The last irreversible stage of inhibition by III is less clear. From the electrophoresis of the low molecular weight fraction after gel filtration (both with and without borohydride reduction), we deduce that the cycloglutamate on the enzyme gradually undergoes ring opening followed by "transimination" to form the oxime of BAGA. A possible mechanism for the ring opening is based on the acylating properties of the carboxyl group as modified in the oxyamide fragment. Thus, cycloglutamates appear to block one of the reactive groups of aspartate aminotransferase by acylation (Khomutov *et al.*, 1967a). The bond between inhibitor and GAD is labile and even such mild work-up as gel filtration on Sephadex led to splitting of the coenzyme and inhibitor from the protein.

Another interesting observation is that gel filtration of complexes of GAD with BAGA or BAP (oximes of the enzyme) and also of the complex of III, EI (24 hr, λ_{max} 380 nm), in all cases led to dephosphorylation of the coenzyme derivative. Reduction by $NaBH_4$ of ES and EI complexes followed by gel filtration also gave dephosphorylated products. On the other hand, the coenzyme cannot be separated from the native holoenzyme by gel filtration, nor is the phosphate split off when the coenzyme is converted to pyridoxamine phosphate by decarboxylative transamination with α-methylglutamate (Huntley and Metzler, 1968). Thus, a labilization of the phospho ester bond takes place for complexes of GAD in which the coenzyme is bound to a compound which partially or wholly occupies the substrate site. This fact suggests that the phosphate group of the coenzyme plays an active role in the transformations of substrate, perhaps by holding the coenzyme and substrate in a proper orientation.

References

Della Pietra, C., Illiano, C., and De Lorenzo, F. (1963), *Biochem. Appl. 10*, 117.

Dunathan, H. C. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 712.

Huntley, T. E. (1970), Ph.D Dissertation, Iowa State University.

Huntley, T. E., and Metzler, D. E. (1968), *in* Symposium on Pyridoxal Enzymes, Yamada, K., Katunuma, N., Wada, H., Ed., Tokyo, Maruzen Co., Ltd., p 81.

Kaneko, T., and Yoshida, R. (1962), *Bull. Chem. Soc. Jap. 35*, 1153; *Chem. Abstracts 57*, 9655*i*.

Khomutov, R. M., Kovaleva, G. K., Severin, E. S., and Vdovina, L. V. (1967a), *Biokhimia 32*, 900.

Khomutov, R. M., Severin, E. S., and Gulyaev, N. N. (1966), *Izv. Akad. Nauk SSSR, Ser. Khim. Nauk*, 1990; *Chem. Abstr. 66*, 85718*a*.

Khomutov, R. M., Severin, E. S., and Gulyaev, N. N. (1967b), *Izv. Akad. Nauk SSSR, Khim. Nauk*, 1622; *Chem. Abstr. 68*, 1288*k*.

Khomutov, R. M., Severin, E. S., and Kovaleva, G. K. (1965), *Dokl. Akad. Nauk SSSR 161*, 1227.

Khomutov, R. M., Severin, E. S., Kovaleva, G. K., Gulyaev, N. N., and Gnuchev, N. V. (1967c), Obmyen Aminokislot, Metzniereba, Tbilisi, p 209.

Khomutov, R. M., Severin, E. S., Kovaleva, G. K., Gulyaev, N. N., Gnuchev, N. V., and Sastchenko, L. P. (1968), *in* Pyridoxal Catalysis: Enzymes and Model Systems, Proceedings of 2nd IUB Symposium, Moscow, Sept 1966, Snell, E. E., Braunstein, A. E., Severin, E. S., Torchinsky, Yu. M., Ed., New York, N. Y., Interscience, p 631.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Neilands, J. B. (1956), *Arch. Biochem. Biophys. 62*, 151.

O'Leary, M. (1969), *Biochemistry 8*, 1117.

Roberts, E., and Frankel, S. (1951), *J. Biol. Chem. 188*, 789.

Sastchenko, L. P., Severin, E. S., and Khomutov, R. M. (1968), *Biokhimia 33*, 142.

Severin, E. S., Gulyaev, N. N., Khurs, E. N., and Khomutov, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 318.

Severin, E. S., Sastchenko, L. P., Kovaleva, G. K., and Khomutov, R. M. (1968), *Biokhimia 33*, 1210.

Shukuya, R., and Schwert, G. W. (1960), *J. Biol. Chem. 235*, 1049.

Umbreit, W. W., and Heneage, P. (1953), *J. Biol. Chem. 201*, 15.

Structure of Schizokinen, an Iron-Transport Compound from *Bacillus megaterium**

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ABSTRACT: Growth of *Bacillus megaterium* ATCC 19213 at limiting concentrations of iron induced the organism to excrete the iron-transport agent schizokinen. Hydrolysis of schizokinen with mineral acid yielded a novel organic hydroxylamine, characterized by synthesis as 1-amino-3-(*N*-hydroxyamino)propane. Oxidation with periodate afforded acetate as the acyl moiety of the hydroxamic acid bonds of schizokinen. Examination of the intact molecule by means of proton magnetic resonance spectroscopy enabled the conclusion that it is composed of a residue of citric acid symmetrically substituted in amide linkage to the amino groups of two residues of 1-amino-3-(*N*-hydroxy,*N*-acetyl)aminopropane. The ferric complex of schizokinen was prepared and shown to be an anion at neutral pH. A minor neutral component of unknown structure named schizokinen A appeared in the medium and could also be prepared from schizokinen by heating.

The technique of derepression of the biosynthesis of microbial iron-transport compounds by culture of various aerobic species at low levels of iron has enabled the isolation of a variety of substances, collectively termed siderochromes, which can be classed chemically as either hydroxamates or phenolates (Neilands, 1971). In the original observation of this phenomenon it was noted that *Bacillus megaterium* excreted a compound believed to be a member of the hydroxamate group (Garibaldi and Neilands, 1956). Later, Lankford

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