Molecular Interactions of Competitive Inhibitors with Bovine Liver Glutamate Dehydrogenase*

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SUMMARY

Four structural analogues of L-glutamate (glutaric acid, thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid) were tested for inhibition as a function of pH, of coenzyme NADP\(^+\) or NAD\(^+\), and of their electronic properties as determined from molecular orbital theory considerations. Plots of \(\frac{v_{\text{max}}}{v}\) and \(\frac{v}{v_{\text{max}}}\) versus pH for the uninhibited and inhibited reaction of the oxidative deamination of L-glutamate with NADP\(^+\) indicated an ionizable group or groups in the enzyme complex with a pK of 7.7 to 7.8. Double reciprocal plots of velocity versus glutamate concentrations in the presence of NADP\(^+\) or NAD\(^+\) showed that each structural analogue of glutamate was a competitive inhibitor with respect to glutamate. NAD\(^+\) increased the affinity of inhibitor for enzyme in comparison with NADP\(^+\). The relative effectiveness of each inhibitor as determined by apparent \(K_i\) values, calculated from Dixon plots, was correlated inversely with the absolute sigma charge density of the "meso" atom of the inhibitor. This suggested that desolvation of this atom may be important for the combination of inhibitor with enzyme. A high degree of solvation as indicated by the magnitude of absolute charge density could have decreased the interaction of inhibitor with enzyme.

Based on the available physical and kinetic evidence for the binding of various ligands to bovine liver glutamate dehydrogenase (L-glutamate:NAD(P)\(^+\) oxidoreductase (deaminating) EC 1.4.1.3), Cross and Fisher (1) proposed a general binding site of 12 x 25 Å containing six subsites labeled I through VI to account for the interactions of substrates, coenzymes, and modifiers with the apoenzyme. Subsite V was designated as the site of attachment for L-glutamate in the active NADPH-glutamate complex. \(V_x\) and \(V_y\) were the binding sites for the \(\alpha\)- and \(\gamma\)-carboxylic groups of L-glutamate. The enzyme requirement for cationic centers 7.5 Å apart for binding glutamate in its extended form was shown earlier by Caughey, Smiley, and Hellerman (2).

Since applications of molecular orbital theory have provided some insight into the molecular events associated with partitioning of aromatic compounds between polar and nonpolar phases (3), it appeared that these techniques might be useful to describe the interactions of small molecules with macromolecules. In the study reported here, the inhibitions by four aliphatic structural analogues of L-glutamate were evaluated as functions of pH, coenzyme, and electronic indices. The latter were obtained from molecular orbital theory considerations of the inhibitors. From this work, a new Subsite \(V_{\text{meso}}\) in addition to Subsites \(V_x\) and \(V_y\) is proposed for interaction of L-glutamate with bovine liver glutamate dehydrogenase.

EXPERIMENTAL PROCEDURE

Bovine liver glutamate dehydrogenase was obtained as a crystalline homogenous protein from Sigma. The enzyme was collected by centrifugation at 4° and was subsequently dissolved in sodium phosphate buffer (0.15 M Na\(_2\)HPO\(_4\) solution adjusted to pH 7.6 with 5 M H\(_2\)SO\(_4\)). After exhaustive dialysis at 4° against buffer, denatured protein was removed from enzyme solutions by centrifugation at 12,000 x g for 20 min at 4°. Stock enzyme solutions (10 mg per ml) were kept refrigerated at 4°. Protein concentrations were estimated by the biuret method (4). Water of 10° ohms resistance was used throughout the experiments.

Reagents—NADP\(^+\), NAD\(^+\), and L-glutamic acid were obtained from Sigma. Thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid were obtained from Aldrich. Glutaric acid was received from Eastman.

Methods—Enzyme assays were performed at 30° as described earlier (5). Velocity was determined as the change in absorbance per min at 340 nm for the reduction of NADP\(^+\) or NAD\(^+\). Apparent inhibition constants \(K_i\) were determined from double reciprocal plots of velocity versus glutamate concentrations and from Dixon plots of reciprocal velocities versus inhibitor concentrations (6). In the former plot \(K_i\) values were calculated from differences in slopes of inhibited versus uninhibited enzyme reactions. In the latter plot \(K_i\) values were determined from the intersection point of lines in the fourth quadrant. Varying the glutamate concentrations by 4-fold provided a large angle for accurate interpolation of \(K_i\). Experimental errors in the determination of \(K_i\) were ±5%.
FIG. 1. Logarithmic plots of $pV_{max}$ and $p(K_n/V_n)$ versus pH for glutamate oxidation in the presence of competitive inhibitor and at a fixed concentration of NADP+. Assays were conducted at 30° in 2.9 ml of 0.1 m Na$_2$HPO$_4$-0.1 m Na$_2$P$_2$O$_7$ buffer adjusted to pH with 5 m H$_2$SO$_4$. The buffer contained enzyme (14.5 µg per ml) and varying amounts of glutamate and inhibitor. The reaction was started by addition of enzyme in buffer. Each point represented the average of duplicate runs similar to those in Fig. 2. The solid circles (●) represent the uninhibited reaction and the open circles (○) represent the inhibited reactions with 1.55 mM glutaric acid or 1.55 mM thiodiglycolic acid. Intersection of lines through experimental points of the plot $pV_{max}$ versus pH gave a value of 7.73; similarly the intersection point of the plot $p(K_n/V_n)$ versus pH gave a value of 7.78 for the control and 7.74 for the inhibited reaction.

Sigma Charge Density—The method of Del Re (7) and Del Re, Pullman, and Yonezawa (8) as modified by Berthod and Pullman (9) was used for the sigma electron system calculations. The parameters employed were those suggested by the references cited. Standard package programs provided by IBM were used for the statistical analyses. All calculations were performed on an IBM 1130 computer. The central or middle atom in the dicarboxylic acid analogues of L-glutamate was designated "meso," i.e. C$_{meso}$, S$_{meso}$, O$_{meso}$, and N$_{meso}$ were the central or middle atoms of glutaric acid, thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid, respectively. The Geneva nomenclature was not applicable for the analogues that contained heteroatoms.

RESULTS

In order to provide an ionic environment (10, 11) for maximum stability during enzyme assay at 30° and to provide suitable buffering potential at each pH examined, 0.1 m Na$_2$HPO$_4$-0.1 m Na$_2$P$_2$O$_7$ solution adjusted with 5 m H$_2$SO$_4$ to the desired pH was used. The enzyme, dissolved in this buffer, remained stable over the pH range 6 to 9 examined. The effect of pH on the rate of oxidation of L-glutamate at a fixed concentration of NADP+ is given in Fig. 1. Plots of $pV_{max}$ and of $p(K_n/V_n)$ as a function of pH showed inflection points at pH 7.7 to 7.8. The negative common logarithm of the variable was designated p. The inflection points indicated the pK of ionizable group or groups involved in the rate-limiting step or steps of the reaction [profile of $pV_{max}$ versus pH] and in the enzyme-coenzyme complex [profile of $p(K_n/V_n)$ versus pH]. The velocity of reaction was too slow for accurate measurement at pH 6 and the velocity was too variable for reliable measurements at pH values greater than 9. The latter result occurred from enzyme inactivation. The initial slopes of $pV_{max}$ versus pH and of $p(K_n/V_n)$ versus pH were 0.5 and 1.0, respectively. Deviation of the slope from whole integers indicated complexity of charge relationships in the rate-limiting step or steps as compared to one-substrate reactions (6). Webb (6) has suggested that such anomalies may be related to the increase in over-all negativity of enzyme with increase of pH.

When a concentration of NAD+ equivalent to that of NADP+ was used with the same buffer, enzyme and glutamate concentrations, inflection points from plots of $pV_{max}$ or $p(K_n/V_n)$ versus pH (not shown) were identical with those previously reported by Greville and Mildvan (12), i.e. pH 7.2 in the plot with $pV_{max}$ and pH 7.9 in the plot with $p(K_n/V_n)$. Examination of the pK values obtained from the NADP+ and NAD+ reac-
tivity phenomena (13) were not observed as doubling or tripling of Fig. 2 and from the Dixon plots of Figs. 3, 4, and 5. Coopera-
tivity constants \( (K_c) \) were calculated from the double reciprocal plots (not shown), did not alter significantly the
portionately the slopes \( (K_m/V_m) \) of inhibition in double
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The inhibition constants \( (pK_i) \) were negative logarithms of \( K_i \) values obtained from the intercepts of the extrapolated lines of the
Dixon plots that are given in Figs. 3, 4, and 5. The values in parentheses were the \( K_i \) concentrations (millimolar) of the inhibitors.
The absolute sigma charge density \( (|Q^+|) \) represented the value calculated from molecular orbital theory for the "meso" atom of the
inhibitor. Values for the single bond-covalent diameter of the "meso" atoms were obtained from reference (6).

| Inhibitor | "Meso" atom | Covalent diameter | Density, \( |Q^+| \) | 0.2 mm | 0.6 mm | 0.2 mm | 0.6 mm |
|-----------|-------------|------------------|----------------|-------|-------|-------|-------|
| HOOCCH_2CH_2COOH (Glutaric acid) | Carbon | 1.54 | 0.086 | 2.9 (1.3) | 3.3 (0.5) | 3.3 (0.5) | 3.6 (0.25) |
| HOOCCH_2SCOOH (Thiodiglycolic acid) | Sulfur | 2.08 | 0.058 | 2.9 (1.3) | 3.3 (0.5) | 3.3 (0.5) | 3.6 (0.25) |
| HOOCCH_2OOCHCOOH (Oxidiglycolic acid) | Oxygen | 1.32 | 0.276 | 2.0 (10) | 2.1 (8) | 2.2 (6) | 2.5 (3) |
| HOOCCH_2NHCH_2COOH (Inimodiacetic acid) | Nitrogen | 1.40 | 0.387 | 1.2 (60) | 1.4 (35) | 1.4 (35) | 1.6 (25) |

The equilibrium constants \( (pK_i) \) and calculated sigma charges \( (|Q^+|) \) are given in Table I. The magni-
itudes indicated that although the rate-limiting step or steps were

1. **Interaction of Competitive Inhibitors with Glutamate Dehydro-
genase**—Glutaric acid has previously been shown to be a com-

petitive inhibitor with respect to glutamate when NADPf was

the coenzyme (2). In view of the kinetic differences with

NADPf instead of NAD+, the inhibition by glutaric acid was re-

examined as a function of pH and coenzyme. In addition,

three structural analogues of glutaric acid, i.e. thiodiglycolic acid,
oxidiglycolic acid, and iminodiacetic acid, were tested for inhibition

(Fig. 2). Double reciprocal plots of velocity versus glutama-

tate concentrations indicated that each of the inhibitors, exam-

ined under the conditions specified, produced a slope \( (K_m/V_max) \)

that intersected with that of the control on the ordinate. This

behavior indicated that each compound was a competitive

inhibitor according to Michaelis-Menten theory (6). The use of

NADPf instead of NADPf as a coenzyme did not alter the

nature of inhibition; the inhibition remained competitive for gluta-

ric acid, thiodiglycolic acid, oxidiglycolic acid, and iminodiacetic

acid. Variation of pH in the presence of NADPf did not alter

the type of inhibition. At equivalent concentrations thiodigly-

colic and glutaric acids provided identical inhibition kinetics

and a plot of \( pV_max \) and \( p(K_m/V_max) \) versus pH for these

compounds is given in Fig. 1. The intersection points on these plots

were almost identical with those values obtained for the unin-

hibited enzyme. Similar plots with the other inhibitors, oxid-

iglycolic acid and iminodiacetic acid (not shown), gave the same

results.

The relative effectiveness of the aliphatic dicarboxylic acids

as inhibitors is summarized in Table I. The apparent inhibition

constants \( (K_i) \) were calculated from the double reciprocal plots of

Fig. 2 and from the Dixon plots of Figs. 3, 4, and 5. Cooper-

ativity phenomena (13) were not observed as doubling or tripling

the effective inhibitor concentrations, although changing propor-

tionately the slopes \( (K_m/V_max) \) of inhibition in double

reciprocal plots (not shown), did not alter significantly the

calculated \( K_i \) values of the inhibitor. The data obtained in the

presence of NADPf or NAD+ were presented as the negative

logarithm of the inhibition constant \( (pK_N^{NADPf} \) or \( pK_N^{NAD+} \))

A more positive value for \( pK_i \) would indicate a greater affinity

of the enzyme for inhibitor if the measured \( K_i \) values represented

the equilibrium constant for dissociation of inhibitor and enzyme

(6). However, the Dixon plots indicated that the calculated \( K_i \)

values probably contained NADPf + or NAD+ terms. Increasing

the coenzyme concentrations from 0.2 to 0.6 mM reduced the

intercept values from which \( K_i \) was calculated in the Dixon plot.

The \( K_i \) values obtained from the Dixon plots and the double

reciprocal plots were in close agreement when the enzyme assays

contained equivalent coenzyme concentrations, e.g. 0.2 mM.

Varying the pH of assay from 6.2 to 8.6 at this coenzyme level

did not alter significantly the apparent \( K_i \) values of the four

inhibitors (see Fig. 1 for glutaric acid and thiodiglycolic acid).

For correlation purposes, it was assumed that the apparent \( K_i \)

values of the competitive inhibitors (Dixon plots) approximated

the equilibrium constants for inhibitor-enzyme interactions at

the higher coenzyme concentration of 0.6 mM. Table I showed

that NADPf increased the apparent affinities of the inhibitors

for enzyme when compared to equivalent concentrations of

NADPf, e.g. the apparent \( K_i^{NADPf} \) value for glutaric acid was

0.5 mM (cf. Reference 2) and the apparent \( K_i^{NAD+} \) value for

glutaric acid was 1.2 mM. The relative effectiveness of the four

structural analogues of L-glutamate as competitive inhibitors

were, in order of decreasing potency, glutaric acid = thiodi-

glycolic acid > oxydiglycolic acid > iminodiacetic acid (Table

1). This relative order of inhibitor effectiveness was not changed

by variation in pH or coenzyme concentration of either NADPf

or NAD+.

**Charge Density and Inhibitor Effectiveness**—The absolute sigma

charge density on the "meso" atom (carbon, sulfur, oxygen, and

nitrogen) of each inhibitor is presented in Table I. The magni-

tude of this parameter has been related to the extent of solvation

of organic compounds in aqueous media (3). The extent of

inhibitory effectiveness was correlated with the magnitude of

the sigma charge with a linear least squares regression equation.

The equations were

\[ pK_{i^{NADPf}} = 3.73 - 5.97 |Q^+| (r = .99) \]
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NAD+ /'

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1

0

6

4

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INHIBITOR (mM)

FIG. 3. Dixon plot of reciprocal velocity against inhibitor (glutaric acid) concentrations in the presence of fixed levels of coenzyme and substrate. Assays were conducted at 30°C in 3.0 ml of 0.1 M NaHPO₄-0.1 M Na₂HPO₄ buffer adjusted to pH 8.10 with 5 M H₂SO₄. The reaction was started by addition of enzyme in buffer to give a final enzyme concentration of 20 µg per ml. Concentrations of coenzyme and substrate were 0.2 mM NADP+ and 0.5 mM glutamate (O), 0.2 mM NADP+ and 2.0 mM glutamate (Δ), 0.6 mM NADP+ and 0.5 mM glutamate (.), 0.6 mM NADP+ and 2.0 mM glutamate (A), 0.2 mM NAD+ and 0.6 mM glutamate (O), 0.2 mM NAD+ and 2.0 mM glutamate (□), 0.6 mM NAD+ and 0.5 mM glutamate (2), and 0.6 mM NAD+ and 2.0 mM glutamate (l). The left ordinate and right ordinate present the reciprocal velocities of the NADP+-glutamate and in the NAD+-glutamate systems. Equivalent concentrations of thiodiglycolic acid to those of glutaric acid gave identical results.

\[ p_{K_{a}}^{NAD^+} = 4.06 - 6.12 | Q^+ | (r = .99) \]  

where \( p_{K_{a}} \) was the negative common logarithm of \( K_{a} \) and \( | Q^+ | \) was the absolute sigma charge density of the “meso” atom of the inhibitors. Equations 1 and 2 represented correlations of inhibitor potency from enzyme systems with a high coenzyme level, 0.6 mM. Data from enzyme systems that contained 0.2 mM coenzyme also gave correlation equations similar to Equations 1 and 2 (not shown). The correlation coefficient \( r \) measured the fit of experimental points to the equation. A value of 1.0 for \( r \) would be a perfect fit. The negative sign of the coefficient of \( | Q^+ | \) indicated that this term was a repulsive quantity in the interaction between enzyme and inhibitor. There was no apparent correlation between inhibitor potency and the \( p_{K_{a}} \) values (14) of the carboxylic acid moieties of the inhibitor.

Negative or positive contributions by the “meso” atoms to the interproton distances between dicarboxylic moieties of the inhibitors were also considered in evaluation of relative inhibitor potency since Caughey et al. (2) showed that this distance governed primarily the extent of enzyme inhibition by a series of alky homologues. Maximum potency of the homologues was recorded for glutaric acid with an interproton distance of approximately 7.45 to 7.65 Å. These values represented the extended form of the acid as determined from its structure in the crystal (2). Variation of ±1.4 Å produced a substantial decrease in the effectiveness of the inhibitor. A survey of the crystallographic literature did not provide information about the conformations of the other three inhibitors (thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid) that were investigated here. An approximation of the “meso” atom effect on the interproton distance was obtained from the covalent diameter for single bonds of the “meso” atoms of the inhibitors. The diameters for the “meso” atoms are given in Table I. With the possible exception of the sulfur atom, the diameters of the oxygen and nitrogen atoms were not different significantly from the diameter of the carbon atom. The sulfur analogue was equally potent in inhibition as the carbon analogue, glutaric acid. Space-filling molecular models showed no apparent relationship between extent of inhibition and the interatomic distance between carboxylic groups, i.e. the inhibitor models were capable of conformations that closely approached the optimal interproton distance of 7.5 Å. Thus, inhibitor activity correlated best with the absolute sigma charge density of the “meso” atom of the inhibitor.

FIG. 4. Dixon plot of reciprocal velocity against inhibitor (oxydiglycolic acid) concentrations in the presence of fixed levels of glutamate and NADP+ or NAD+. Assays were conducted at 30°C in 3.0 ml of 0.1 M NaHPO₄-0.1 M Na₂HPO₄ buffer adjusted to pH 8.10 with 5 M H₂SO₄. The reaction was started by addition of enzyme in buffer to give a final enzyme concentration of 20 µg per ml. Concentrations of coenzyme and glutamate were 0.2 mM (O), 0.2 mM and 2.0 mM (Δ), 0.6 mM and 0.5 mM (.), 0.6 mM and 2.0 mM (A), respectively. The coenzymes used in experiments represented by the top and bottom sections of the figure were NAD+ and NADP+, respectively. The solid line intercepts in the fourth quadrants indicate the values of \( K_{i} \) for the competitive inhibitor oxydiglycolic acid in the NAD+-glutamate and NADP+-glutamate systems.
DISCUSSION

Four compounds very similar in structure were shown to be competitive inhibitors of bovine liver glutamate dehydrogenase. The compounds competed with L-glutamate for combination with the enzyme. Varying the pH did not change the inhibition mechanism; it remained competitive. Substitution of NADPH for NAD+ decreased the extent of inhibition by the analogues.1 Furthermore, varying the pH of the uninhibited and inhibited enzyme in the presence of NADPH showed that an ionizable group with a pK_a of about 7.8 was present in the NADPH-enzyme complex. It was tempting to speculate that the ionizable group of glutamic acid bound to the coenzyme-enzyme complex existed in part in the active site for binding glutamate. Then by inference, the "meso" atom of the competitive inhibitors (glutaric acid, thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid) may be involved in the combination of inhibitor with the active site for binding glutamate. Then by inference, the "meso" atom of glutamate may also be required for combination with glutamate dehydrogenase.

REFERENCES


* Consideration (3) of solute-solvent interactions from an experimental and a molecular orbital theory point of view indicated that partitioning of a compound into an aqueous phase was "charge controlled" (\(Q^*\)), while partitioning into nonpolar phase was "polarizability controlled" (SF). It was the relative contribution of each controlling factor that determined the partition coefficient observed for a compound. Recently these parameters were used for correlations of inhibitor activity in biological systems (21, 22).
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