The Reaction of Amino-oxyacetate with Pyridoxal Phosphate-Dependent Enzymes

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The carbonyl reagent amino-oxyacetate is frequently used in metabolic studies to inhibit individual pyridoxal phosphate enzymes. The reaction of this compound with three such enzymes, aspartate transaminase, 4-aminobutyrate transaminase and dopa (3,4-dihydroxyphenylalanine) decarboxylase, was studied to determine the extent to which the inhibition is reversible and the rates at which it takes place. Reactions were followed by observing changes in the absorption spectra of the bound coenzyme and by measuring loss of enzyme activity. The reactions with aspartate transaminase and aminobutyrate transaminase were not rapidly reversible and had second-order rate constants (21°C) of $400 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1300 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively and at all concentrations studied showed the kinetics of a simple bimolecular reaction. The reaction with 4-aminobutyrate transaminase could not be reversed and that with aspartate transaminase could only be reversed significantly by addition of cysteinesulphonate to convert the enzyme into its pyridoxamine form. The first-order rate constant (21°C) for the reverse reaction was $4 \times 10^{-5} \text{ s}^{-1}$. Dopa decarboxylase inhibition by amino-oxyacetate was more rapid and more readily reversible, but measurements of rate and equilibrium constants were not obtained for this enzyme.

Amino-oxyacetate has been used frequently in metabolic studies with the intention of singling out one enzyme for inhibition. Much of this work has been aimed at inhibiting brain 4-aminobutyrate transaminase (EC 2.6.1.19) to increase concentrations of the neurotransmitter 4-aminobutyrate (e.g. Baxter & Roberts, 1961; Osuide, 1972). The general reactivity of amino-oxyacetate towards pyridoxal phosphate-dependent enzymes is well recognized, since it is known that the compound reacts with 4-aminobutyrate transaminase (Wallach, 1961; Baxter & Roberts, 1961; Wu, 1976), alanine transaminase (EC 2.6.1.2; Hopper & Segal, 1962), glutamate decarboxylase (EC 4.1.1.15; Roberts & Simonsen, 1963), alanine racemase (EC 5.1.1.1; Free *et al.*, 1967), histidine decarboxylase (EC 4.1.1.22; Leinweber, 1968), D-amino acid transaminase (EC 2.6.1.6; Yonaha *et al.*, 1975) and cystathionase (EC 4.4.1.1; Beeler & Churchich, 1976). Amino-oxyacetate is also widely used to block aspartate transaminase (EC 2.6.1.1) during metabolic studies where it is intended to be specific (Rognstad & Clark, 1974; Longshaw *et al.*, 1972; Williamson *et al.*, 1971).

Many studies *in vitro* have been made on the mechanism of amino-oxyacetate inhibition of various enzymes, e.g. alanine transaminase (Hopper & Segal, 1962), pyridoxal kinase (EC 2.7.1.35; Davanzo *et al.*, 1966), glutamate decarboxylase (Roberts & Simonsen, 1963) and 4-aminobutyrate transaminase (Wu, 1976). In these studies the inhibition is treated as though it were of the rapidly reversible type, classically divided into competitive, non-competitive, uncompetitive etc. In these experiments amino-oxyacetate was included with substrates, enzyme and very often pyridoxal phosphate, with which it reacts, for periods of 5–60 min and then the amount of product was used in the construction of reciprocal plots as though it represented initial velocity. In one case the apparent inhibition of pyridoxal kinase (Davanzo *et al.*, 1966) is clearly due to a stoichiometric reaction with the substrate. Other workers (e.g. Karpeisky *et al.*, 1963) have attempted quantification of the efficacy of this and other carbonyl reagents by finding the concentration at which half the enzyme activity remains after various times (I_{50}).

The present work is intended to investigate the inhibition by amino-oxyacetate of three pyridoxal phosphate enzymes to examine the basis for claims to specificity and to find the extent to which the inhibition might be considered rapidly reversible.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

The enzymes examined were aspartate transaminase, 4-aminobutyrate transaminase and dopa (3,4-dihydroxyphenylalanine) decarboxylase (EC 4.1.1.28).

Experimental

Chemicals

Aspartic acid, 2-oxoglutaric acid, amino-oxyacetic acid, hydroxylamine, 2-mercaptoethanol, cysteine-sulphinic acid and dopa were bought from Sigma (London) Chemical Co., London S.W.6, U.K. Solutions of aspartic acid, 2-oxoglutaric acid, amino-oxyacetic acid and cysteine-sulphinic acid were neutralized with NaOH before use. Dithiothreitol and pyridoxal phosphate were bought from Koch-Light, Colnbrook, Bucks, U.K. Hepes was bought from Hopkin and Williams, Chadwell Heath, Essex, U.K. Sephadex and QAE-Sephadex [diethyl-(2-hydroxypropyl)aminoethyl-Sephadex] were from Pharmacia (G.B.), Uxbridge, London W.5, U.K., and ion-exchange cellulose materials were from Whatman Biochemicals, Maidstone, Kent, U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K. Solutions of amino-oxyacetate were prepared just before use.

Enzymes

Aspartate transaminase was purified from pig heart by the modification described by Martinez-Carrion *et al.* (1967) of the method of Jenkins *et al.* (1959). 4-Aminobutyrate transaminase was prepared from rabbit brain as described by John & Fowler (1976).

Pig kidney dopa decarboxylase was prepared by homogenizing the cortex of ten kidneys in 2 vol. of 0.14M-NaCl with a Waring blender. After centrifuging (3.2×10^6 g-min), the supernatant was treated with pyridoxal phosphate (final concn. 0.1 mM) and 140g of $(\text{NH}_4)_2\text{SO}_4$ added per litre of supernatant. After centrifuging (150000g-min), the precipitate was discarded and to each litre of clear supernatant was added 330g of $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained after centrifuging (150000g-min) was dissolved in 10mM-sodium phosphate buffer, pH7.2, containing 10 μ M-pyridoxal phosphate and subjected to the column fractionations described below. Between each fractionation, active fractions were precipitated by adding 450mg of $(\text{NH}_4)_2\text{SO}_4$ /ml.

(1) *DEAE-cellulose*. A column (4.3cm $\times$ 30cm) was equilibrated with 10mM-sodium phosphate buffer containing 10 μ M-pyridoxal phosphate, 0.1 mM-EDTA and 1 mM-2-mercaptoethanol, pH7.2, and developed with a linear gradient from equilibration buffer to 0.2M-NaCl in the same buffer; total volume used was 1 litre.

(2) *QAE-Sephadex*. A column (4.3cm $\times$ 15cm)

was equilibrated with 80mM-sodium phosphate buffer containing the same additions as for fractionation (1) and developed with a linear gradient from equilibration buffer to 0.2M-NaCl in the same buffer; total volume used was 2 litres.

(3) *DEAE-cellulose*. A column (1.6cm $\times$ 15cm) was equilibrated with 5mM-sodium phosphate containing 0.1 mM-dithiothreitol, pH6.5, and developed with a linear gradient from starting buffer to 0.5M-NaCl in the same buffer.

(4) *QAE-Sephadex*. A column (1.6cm $\times$ 8cm) was equilibrated with 5mM-sodium pyrophosphate buffer containing 1 mM-2-mercaptoethanol, 0.1 mM-EDTA and 70mM-NaCl, pH8.5, and developed with a linear gradient from starting buffer to the same buffer containing 0.4M-NaCl; total volume used was 500ml.

(5) *Sephadex G-100*. A column (2.2cm $\times$ 85cm) was equilibrated with 10mM-sodium phosphate containing 0.1 mM-dithiothreitol, pH6.8.

The procedure is a modification of one given by Professor Carlo Turano (personal communication).

Enzyme assays

Aspartate transaminase was measured by the method of Karmen (1955) by using conditions described by John & Fasella (1969). 4-Aminobutyrate transaminase was measured by the method of Salvador & Albers (1959) and dopa decarboxylase as described by Charteris & John (1975).

Spectrophotometry

U.v. and visible absorption spectra were determined by using either a Unicam SP. 800 or a Beckman model 25 recording spectrophotometer, and the latter was also used to follow the course of the slower reactions. Faster reactions were followed with a Durrum-Gibson stopped-flow spectrophotometer.

Results and Discussion

Spectral changes resulting from combination of amino-oxyacetate with aspartate transaminase

The combination of amino-oxyacetate with aspartate transaminase is accompanied by a change in the absorption spectrum of enzyme-bound pyridoxal phosphate. The peak at 360nm attributed to the aldimine formed between the coenzyme and a lysine residue (Braunstein, 1964; Guirard & Snell, 1964) shifts to 385nm, as shown in Fig. 1(a). This may be attributed to substitution by the amino group of amino-oxyacetate to form an oxime. At the concentrations used in this experiment the reaction is relatively slow but apparently irreversible, so that the compound can be used as an effective titrating agent, as shown in Fig. 1(b).

Fig. 1. Spectral changes accompanying reaction between aspartate transaminase and amino-oxyacetate

Aspartate transaminase (1.1 ml, 85 μM) was treated with successive portions (5 μl) of 6.2 mM-amino-oxyacetate. An absorption spectrum was recorded when reaction was complete after each addition. The original spectrum, that showing lowest absorption at 400 nm, and those observed after each of three additions are shown in (a). Total changes in A_{400} for an experiment that was identical except that 5 mM-amino-oxyacetate was used are also shown in (b). In this case several more portions of amino-oxyacetate were added after reaction was complete. The reactions were carried out at 21.6°C in 0.04 M-Hepes adjusted to pH 8.0 with NaOH.

Course of the reaction between aspartate transaminase and amino-oxyacetate

Data obtained from each of the separate reactions used to give the spectra shown in Fig. 1 were plotted to give the second-order rate constant as shown in Fig. 2. Under these conditions, therefore, the simplest interpretation is that the reaction is an irreversible bimolecular process with a rate constant of $4.2 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 21.6°C.

At higher concentrations of amino-oxyacetate, because the reaction becomes too fast for conventional mixing methods, the stopped-flow method for rapid mixing was used. Fig. 3 shows the course of the reaction when amino-oxyacetate concentration was 20 mM and the enzyme concentration was 0.06 mM. A semilogarithmic plot showed the reaction to be first order for at least 95% of its course, and this was also true for the reactions at other inhibitor concentrations, the pseudo-first-order constants for which

Fig. 2. Kinetics of the reaction between aspartate transaminase and amino-oxyacetate

The course of each of the reactions used to give the spectra of Fig. 1 was followed and data taken from the curves were replotted to give the second-order rate constant. Points (●) from the reaction after the first addition of amino-oxyacetate were obtained by using values of 85 μM for the enzyme concentration and 31 μM for the amino-oxyacetate concentration as a_0 and b_0 respectively in the expression shown on the ordinate. The value x represents the amount of product at any time. Points derived for the subsequent reactions (▲) and (■) were obtained by taking the enzyme concentration to be 54 and 23 μM respectively, with the amino-oxyacetate at 31 μM in each case.

Fig. 3. Course of the reaction between aspartate transaminase and high concentrations of amino-oxyacetate. Oscilloscope trace from a stopped-flow experiment showing decreasing transmission at 400 nm when aspartate transaminase (60 μM) reacts with amino-oxyacetate (20 mM). Both reactants were dissolved in 0.04 M-Hepes adjusted to pH 8.0 with NaOH. The temperature was 21°C. The upper trace shows the course of the reaction and the lower trace shows the end point determined by triggering a sweep 1 min after the end of the first sweep.

Fig. 4. Variation of observed first-order rate constant with amino-oxyacetate concentration

The first-order rate constant ($k_{\text{obs.}}$) for a series of reactions like that shown in Fig. 3 were obtained by using the concentrations of amino-oxyacetate shown on the abscissa.

are plotted against amino-oxyacetate concentration in Fig. 4. The second-order rate constant determined from the slope of this line is $3.9 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (21°C), in good agreement with that obtained in the earlier experiments.

The point under examination here is whether initial binding, such as might be expected from ionic interaction between the carboxy group of the amino-oxyacetate and a positive centre on the enzyme normally used to bind substrate, is sufficiently strong that significant concentrations of a reversible complex are formed as in eqn. (1):

The linear dependence of rate constant with $[\text{I}]$ and the constant second-order rate constant are what one would expect for a simple bimolecular reaction as in eqn. (2):

but would also be true for eqn. (1) when the dissociation constant K is very high compared with the inhibitor concentration. Thus the evidence shows that if the carboxy group has any directive effect it is insufficient under these conditions to give a significant concentration of a reversible complex.

Reversibility of amino-oxyacetate inhibition of aspartate transaminase

Despite the apparently irreversible nature of the reaction between amino-oxyacetate and aspartate transaminase, fully active enzyme can be regenerated by coupling with an even more 'irreversible' reaction. Cysteinesulphinat is a substrate for aspartate transaminase and reacts with the aldimine form (E_L) to give the amine form (E_M). The keto acid formed from cysteinesulphinat in this process breaks down and makes the conversion of E_L into E_M virtually irreversible (Jenkins & D'ari, 1966):

So long as the cysteinesulphinat reaction is relatively fast, the rate of E_M formation, which can be followed by observing the peak at 330nm, will be a measure of the rate of dissociation of amino-oxyacetate from E_L . In a separate experiment with the same concentrations of native enzyme ($85 \mu\text{M}$) and cysteinesulphinat (2.2 mM) the spectral change was found to be complete within the mixing time of 10s, showing that the conversion of E_L into E_M by cysteinesulphinat is very fast relative to the rate of the spectral changes shown in Fig. 5. The further observation that the rate of the slow reaction is unaffected by cysteinesulphinat concentration supports the proposed mechanism.

Fig. 5. Spectral changes occurring when cysteinesulphinat reacts with the aspartate transaminase-amino-oxyacetate complex

The sample of enzyme-amino-oxyacetate complex (1.1 ml , $85 \mu\text{M}$) formed in the experiment of Fig. 1(a), in which stoichiometric amounts of the two reactants had been mixed and left for several hours, was treated with $5 \mu\text{l}$ of 0.5 M -cysteinesulphinat and the absorption spectrum recorded at intervals. The spectrum with the highest absorbance at 385nm was recorded 16min after addition of cysteinesulphinat and the remainder were obtained at the times indicated in Fig. 6.

We conclude therefore that the rate of increase in A_{330} and the rate of recovery of enzyme activity, which run together as shown in Fig. 6, are a measure of the slow rate of dissociation of amino-oxyacetate from the aldimine form of the enzyme. The continuous line through the point of Fig. 6 is theoretical, constructed for a first-order process occurring with a rate constant of $4.0 \times 10^{-5} \text{ s}^{-1}$ with $\Delta A_{385} = 0.55$ and specific activity of 130 units/mg (where a unit of activity is defined as the amount of enzyme required to convert $1 \mu\text{mol}$ of substrate/min) as the respective end-points. By using this value for the rate constant for dissociation and the value of $4.2 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the association rate constant given above a dissociation equilibrium constant of 95 nM can be calculated.

Spectral changes resulting from combination of amino-oxyacetate with 4-aminobutyrate transaminase

The combination of amino-oxyacetate with 4-aminobutyrate transaminase gives a less marked spectral shift than with aspartate transaminase (Fig. 7a) and the reaction is thus more readily examined by difference spectroscopy (Fig. 7b). Fig. 8(a) shows the results of a titration of 4-aminobutyrate transaminase with amino-oxyacetate by using difference spectroscopy to follow the changes occurring. After each addition the reaction was observed until it had stopped before the measure-

ment was made. The concentration of enzyme determined from this spectral titration ($9.2 \mu\text{M}$) does not equal the concentration calculated from the protein concentration and molecular weight ($26 \mu\text{M}$). This disparity is not due to any obvious contamination with other proteins, and it seems likely that only a certain amount of the enzyme can react with amino-oxyacetate. Presumably only this part of the enzyme reacts with substrates and it is possible that the remainder has undergone a partial inactivation process through aging similar to that seen with aspartate transaminase (Martinez-Carrion *et al.*, 1967).

Titration of 4-aminobutyrate transaminase with amino-oxyacetate by using enzyme activity as an indicator of the reaction

Fig. 8 shows that equal amounts of enzyme activity are lost with each successive addition and that this linear dependence continues until activity loss is complete. This observation and the linear spectral titrations show that at these concentrations the reaction is essentially irreversible. Calculations from the data of Fig. 8 give the concentration of enzyme that reacts with amino-oxyacetate as $12.5 \mu\text{M}$, whereas calculations using protein concentration and molecular weight (116000; John & Fowler, 1976) give an enzyme concentration of $32 \mu\text{M}$.

The non-reactivity of a certain amount of the enzyme towards amino-oxyacetate has been observed with other preparations of the enzyme, and on occasion the proportion of reactive enzyme has been very close to 50%. It is possible that the enzyme has 'half-of-the-sites reactivity' (Levitzki *et al.*, 1971), but it seems more probable that loss of activity is progressive and the occasional approximation to 50% is coincidental.

Course of the reaction between amino-oxyacetate and 4-aminobutyrate transaminase

The results of the time-dependent inhibition of 4-aminobutyrate transaminase by amino-oxyacetate at 25°C , 37°C and in the presence of 10 mM -L-4-aminobutyrate are shown in Fig. 9. The second-order constants are $1.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (25°C), $2.7 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (37°C) and $3.5 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (37°C with 10 mM -4-aminobutyrate).

Extensive dialysis did not restore the activity of the inhibited enzyme even if pyridoxal phosphate (0.1 mM) was included in the dialysis buffer. It is possible that the inhibition may be reversible, but dissociation must be very slow. In this case, unlike with aspartate transaminase, a compound like cysteinesulphinatate is not available, so that the reversibility of the reaction cannot so readily be examined.

Fig. 6. *Re-activation of aspartate transaminase by cysteinesulphinatate*

The solution of enzyme-amino-oxyacetate complex treated with cysteinesulphinatate (Fig. 5) was examined for enzyme activity by removing samples ($20 \mu\text{l}$) and diluting to 1 ml in water before assay ($\bullet$). Changes in absorbance are also shown ($\circ$). The continuous line is theoretical for a first-order process having the constants described in the text.

Fig. 7. Spectral changes occurring when 4-aminobutyrate transaminase reacts with amino-oxyacetate. The enzyme (0.4 ml; 1.5 mg/ml in 0.1 M-sodium acetate, pH 5.5) was treated with 2 μ l of 8 mM-amino-oxyacetate (a). The spectrum with the highest peak at 405 nm is that of the native enzyme and the other is that recorded a few minutes after addition of the amino-oxyacetate. No further change occurred with time or with further addition of amino-oxyacetate. The difference spectra (b) were obtained by adding two successive portions (2 μ l) of 0.8 mM-amino-oxyacetate to 0.4 ml of enzyme (1.5 mg/ml in 0.1 M-sodium acetate buffer, pH 5.5.).

Fig. 8. Titration of 4-aminobutyrate transaminase with amino-oxyacetate. The enzyme (0.4 ml; 1.5 mg/ml) was titrated by adding successive portions (2 μ l) of 0.8 mM-amino-oxyacetate.

Course of reaction between 4-aminobutyrate transaminase and hydroxylamine

Fig. 9 shows the second-order plot for the reaction between 4-aminobutyrate transaminase and hydroxylamine. The rate constant of $1.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (37°C) is about one-half of that with amino-oxyacetate, showing that the carboxy group has very little effect on the rate of inhibition.

Reaction of amino-oxyacetate with dopa decarboxylase

The absorption spectra resulting from the reaction of dopa decarboxylase with amino-oxyacetate are

The A_{428} was observed until constant and the change in A_{428} used to construct the titration curve (●). In other experiments (enzyme concentration 1.9 mg/ml) the extent of reaction was monitored by measuring loss of activity. The points show activity remaining after 90 min (■) and 5 h (▲) treatment with amino-oxyacetate.

Fig. 9. Reaction of carbonyl reagents with 4-aminobutyrate transaminase

Samples of the enzyme were removed after treatment with carbonyl reagent and diluted by addition to substrate solution. Dilution was sufficient to make negligible further reaction of the carbonyl reagent during the assay. The conditions used were: (○) amino-oxyacetate, 2.5 μ M, 37°C; (△) amino-oxyacetate, 2.5 μ M, 25°C; (□) amino-oxyacetate, 2.5 μ M, 37°C, in the presence of 10 mM-4-aminobutyrate; (●) hydroxylamine, 4.5 μ M, 37°C. All reactions were carried out in 25 mM-acetic acid adjusted to pH 8.2 by adding solid Tris. The enzyme concentration used was 1.1 μ M, determined by titration with amino-oxyacetate. The values a_0 , b_0 and x represent the concentrations of enzyme, carbonyl reagent and product respectively.

more complicated than those for the other two enzymes. Fig. 10 shows the spectrum of the native enzyme and the spectra obtained after several additions of amino-oxyacetate. The first addition (24 μ M) produced a decrease at 420 nm, but further additions produced no further decrease at this wavelength. An estimate of the enzyme molarity was made by determining the amount of pyridoxal phosphate released by 0.1 M-NaOH and using the known absorption coefficient of the free coenzyme under these conditions (Peterson & Sober, 1954), and the value determined was 26 μ M. The reaction between the 420 nm-absorbing form of the enzyme and amino-oxyacetate appeared to go to completion as measured at this wavelength. Although no value for the rate constant can be given, because the reaction was complete within the time needed to record the spectrum, it is clearly much higher than those for the other two enzymes. By assuming that the reaction was at least 90% complete within 10 s a lower limit for the rate constant was estimated to be $2.5 \times$

Fig. 10. Spectral changes occurring when dopa decarboxylase reacts with amino-oxyacetate

The enzyme (0.9 ml; 33 μ B in 0.05 B-Tris/HCl, pH 7.5) was treated with 5 μ l of 4.3 mM-amino-oxyacetate and the spectrum recorded after 5 min: 10 min after addition of a further 5 μ l of the same amino-oxyacetate, another spectrum was recorded. Spectra were also drawn 30 min after each of the subsequent 10 μ l additions; the second of these was of 23 mM-amino-oxyacetate. After each spectrum had been drawn two 5 μ l samples were removed for assay of enzymic activity. The spectrum of native enzyme is that with the peak above 400 nm and the spectra with increasing absorptions at 390 nm are those obtained after addition of successive portions of amino-oxyacetate solution. The lowest line is the baseline obtained by using the buffer solution alone.

$10^{-1} \text{ M}^{-1} \cdot \text{s}^{-1}$. However, although further addition of amino-oxyacetate produced no further decrease at 420 nm, the increase at 385 nm observed with the first addition continued for two more additions of amino-oxyacetate to a total concentration of 72 μ M. It is possible that this is caused by reaction with coenzyme bound in a different way but also forming the oxime.

Removal of a sample of the enzyme after the first addition of amino-oxyacetate gave an activity

measurement, from a 5 min incubation, which was 20% of that of the untreated enzyme. The value obtained after the second addition of amino-oxyacetate was still 10% that of the untreated enzyme. The most reasonable explanation of this is that the 50-fold dilution of the sample that occurs in adding it to the assay solution lowers the concentration sufficiently to allow a certain amount of reversal. If the dissociation is rapid, these data would be accounted for by a dissociation constant of $0.2 \mu\text{M}$, ignoring any effect of the substrate. The reaction between dopa decarboxylase and amino-oxyacetate, although not as well characterized as those for the other two enzymes, is clearly much faster and more readily reversible.

For two of the enzymes studied in this work, inhibition may be considered complete so long as an amount of amino-oxyacetate is used that is at least stoichiometric with the enzyme. This is also true for cystathionase (Beeler & Churchich, 1976), which appears to be the only other enzyme that has been investigated from this point of view. It seems reasonable to suppose that oxime formation between amino-oxyacetate and many other pyridoxal phosphate-dependent enzymes is similarly complete and that its use as a selective inhibitor is unwise. However, the three enzymes for which data are available show differences of rates of reaction, which, although not great, would confer some kinetic specificity.

The molecular basis for the observed differences in rate constant is difficult to ascertain. The rate constant for the reaction of amino-oxyacetate with pyridoxal phosphate is $4.8 \text{M}^{-1} \cdot \text{s}^{-1}$ (25°C) (Beeler & Churchich, 1976). The reaction with the internal aldimine that, because of the absorption spectra and by analogy with other pyridoxal phosphate-dependent enzymes, may be presumed to exist between the coenzyme and a lysine residue of these enzymes, would be expected to be much faster than that with the free aldehyde simply because of the greater reactivity of aldimines compared with the free aldehydes (Jencks & Cordes, 1963).

Jencks & Cordes (1963) also showed that protonated aldimine reacts faster than the unprotonated form, but it is not possible to tell from their data what the second-order constants for the transaldimination reactions are, because in their experiment only catalytic steady-state concentrations of aldimine were present. It is clear, however, that the rate constant of $61 \text{M}^{-1} \cdot \text{s}^{-1}$ for cystathionine and amino-oxyacetate (Beeler & Churchich, 1976) can easily be reached without any directive effects caused by interaction between the carboxy group of the inhibitor and the enzyme, since this value comes within the range of values reported by Jencks & Cordes (1963). The values for aspartate transaminase ($420 \text{M}^{-1} \cdot \text{s}^{-1}$ at 22°C) and 4-aminobutyrate transaminase, ($1300 \text{M}^{-1} \cdot \text{s}^{-1}$ at 25°C), are higher than anything found by

Jencks & Cordes (1963), but this is probably because the proportion of aldimine present in their system was very low at the concentrations of pyridoxal phosphate and base that they used. The view that the carboxy group of amino-oxyacetate has little directive effect in these reactions is borne out by the observation that the compound reacts only twice as fast as hydroxylamine with 4-aminobutyrate transaminase.

The measurement of apparent dissociation constants (Wallach, 1961; Wu, 1976) for amino-oxyacetate and 4-aminobutyrate transaminase by classical steady-state methods is clearly not feasible for this slowly reacting irreversible inhibitor. At concentrations in the region of $1 \mu\text{M}$ used in these earlier studies the half-time for inactivation in the absence of substrate would be about 5 min, so that inactivation would be occurring during the incubation. Increasing substrate concentrations increase the product concentration not only by saturating the enzyme more but also by slowing inactivation. When, by chance, incubation times and inactivation times are roughly equal and the resulting product concentrations are mistakenly plotted as initial velocities, the patterns obtained resemble those for classical competitive inhibition. Dissociation constants derived from such treatment are obviously spurious.

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