Enzyme-Induced Inactivation of Transaminases by Acetylenic and Vinyl Analogues of 4-Aminobutyrate

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(Received 25 August 1978)

The reactions of two analogues of 4-aminobutyrate, namely 4-aminohex-5-ynoate and 4-aminohex-5-enoate, with three transaminases were studied. Three pure enzymes were used, aminobutyrate transaminase (EC 2.6.1.19), ornithine transaminase (EC 2.6.1.13) and aspartate transaminase (EC 2.6.1.1), and the course of the reactions was studied by observing changes in the absorption spectrum of the bound coenzyme and by observing loss of activity. All of the enzymes were inactivated by either inhibitor, but aminohexenoate showed a marked specificity for aminobutyrate transaminase. Aminohexynoate was most potent towards ornithine transaminase, and with this enzyme transamination of the inhibitor is an important factor in protecting the enzyme. Most of the reactions could be analysed as first order, with the observed rate constant showing a hyperbolic dependence on inhibitor concentration.

Ideally compounds intended to act as drugs by specific inhibition of a particular metabolic step should show an absolute selectivity towards the target enzyme. One means of approaching this ideal is to prepare a compound that not only bears a strong structural resemblance to the substrate, but also becomes reactive as an irreversible inhibitor only as a result of the operation of the enzyme's normal mechanism. The pharmacological potential of such enzyme-induced irreversible inhibition has been pointed out (Fasella & John, 1969; Rando, 1974a) and inhibitors of this type have been referred to as ' k_{cat} inhibitors' (Rando, 1974a) and 'suicide enzyme inactivators' (Abeles & Maycock, 1976).

Several inhibitors of this type have been synthesized with the intention of selectively inhibiting 4-aminobutyrate transaminase (EC 2.6.1.19) (Fowler & John, 1972; Jung & Metcalf, 1975; Lippert *et al.*, 1977). The inhibition of this enzyme by a naturally occurring compound, gabaculine (5-amino-1,3-cyclohexadienyl carboxylic acid), is also by an enzyme-induced mechanism (Rando & Bangerter, 1977). Aminobutyrate transaminase has received attention because its substrate is an inhibitory neurotransmitter, and therefore its selective inhibition is pharmacologically interesting.

The reactions of pyridoxal phosphate-dependent enzymes with substrate analogues of this kind may be complex, as is shown by the reaction of the glutamate analogue, serine sulphate, with aspartate transaminase (John & Fasella, 1969). In this case the enzyme catalyses the elimination of sulphate from the β -position, and there is evidence indicating that

the resulting aminoacrylic intermediate reacts with lysine-258 and cysteine-390 of the enzyme (John *et al.*, 1973). However, in addition there is a slow transamination that protects the enzyme from further inactivation, because the pyridoxamine form of the enzyme, although fully active in the normal transamination reaction, cannot combine productively with amino acids. If inhibition is investigated without regard to the possibility of a protecting transamination it could be wrongly concluded that a compound does not inhibit a particular enzyme. Furthermore, the goal of very high specificity, which the enzyme-induced type of inhibition might be expected to approach, may be made less easily attainable because of the existence of families of enzymes that share the same catalytic mechanism.

Two compounds, namely 4-aminohex-5-ynoic acid and 4-aminohex-5-enoic acid, which might be considered as acetylenic and vinyl derivatives of 4-aminobutyrate, have been prepared as enzyme-induced inhibitors of aminobutyrate transaminase (Jung & Metcalf, 1975; Lippert *et al.*, 1977). They inactivate the enzyme, and the mechanism proposed is one suggested for the inactivation of aspartate transaminase by 2-aminobut-3-enoic acid (Rando, 1974b), in which the enzyme induces reactivity by introducing conjugation of double bonds. The inhibitors were also shown, by measuring rates of loss of activity, to be relatively inert towards impure preparations of some other pyridoxal enzymes.

The present paper is an account of investigations into the reactions of these compounds with some transaminases. The enzymes used were all purified

to homogeneity and the study depends heavily on observation of changes in the absorption spectrum of the bound coenzyme. As well as aminobutyrate transaminase, aspartate transaminase (EC 2.6.1.1) and ornithine transaminase (EC 2.6.1.13) were studied, the last particularly because, like aminobutyrate transaminase, it catalyses transamination of amino groups on carbon atoms that bear no carboxy group.

Experimental

Enzymes

Experiments with aspartate transaminase were carried out with the pure α -subform of the pig heart cytoplasmic enzyme prepared by the method of Martinez-Carrion *et al.* (1967). Ornithine transaminase was prepared from rat liver by the method of Peraino *et al.* (1969) and 4-aminobutyrate transaminase from rabbit brain by the method of John & Fowler (1976). Enzyme concentrations are expressed as concentration of bound pyridoxal phosphate and were determined by using $\epsilon_{280} = 7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for aspartate transaminase (Birchmeier *et al.*, 1973), $\epsilon_{412} = 5.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ornithine transaminase (G. M. Bridge & R. A. John, unpublished work) and $\epsilon_{415} = 1.2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for aminobutyrate transaminase (L. J. Fowler & R. A. John, unpublished work). The concentration of the last enzyme was also determined by titration with amino-oxyacetate (John *et al.*, 1978).

Chemicals

The inhibitors 4-amino-5-hexynoic acid and 4-amino-5-hexenoic acid were a gift from Merrell International Research Centre, Strasbourg, France. Tris (Puriss grade) and 2-oxoglutaric acid were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Aspartic acid, ornithine and 4-aminobutyrate were from Sigma (London) Chemical Co., London SW6, U.K. Other chemicals used were supplied by BDH Chemicals, Poole, Dorset, U.K.

Enzyme activity assays

Aspartate transaminase was assayed by the method of Karmen (1955), ornithine transaminase by the method of Peraino & Pitot (1963) and aminobutyrate transaminase by the method of Salvador & Albers (1959).

Absorption spectra

Absorption spectra were determined on a Beckman model 25 recording spectrophotometer.

Determination of kinetic constants

When, graphically, the observed first-order rate constants for inhibition ($k_{\text{obs.}}$) were found to vary hyperbolically with inhibitor concentration, the results were taken to indicate the initial formation of significant amounts of a rapidly reversible complex. The dissociation constant, K , for complex formation, and the first-order rate constant, k , for irreversible inhibition were estimated using a weighted least-squares linear-regression analysis. Simple errors in $k_{\text{obs.}}$ were assumed and weights of $k_{\text{obs.}}^2/[I]^2$ were used. The method used was that described by Wilkinson (1961) except that $k_{\text{obs.}}$ was substituted for v , k for $V_{\text{max.}}$, $[I]$ for $[S]$ and K for K_m . The data were handled by a Hewlett-Packard 9820 A programmable calculator.

Results and Discussion

The results of the experiments on all three enzymes and with both substrates will be interpreted in terms of Schemes 1(a) and 1(b) which are based on the classical Snell-Braunstein mechanism for transamination (Braunstein, 1964; Guirard & Snell, 1964). Inactivation will be presumed to occur as a result of reaction of a nucleophile in the enzyme with either or perhaps both of the reactive intermediates E' and E_kI' . Several plausible routes are possible all leading effectively to the same result, namely covalent binding of the enzyme-induced inhibitor to the enzyme protein.

Reactions of aminobutyrate transaminase

The changes in coenzyme absorption spectrum that are seen when aminobutyrate transaminase reacts with aminohexynoate occur in two phases and the rates of each phase are dependent on inhibitor concentration. Fig. 1 shows the changes that occur when the enzyme is treated with 0.63 mM-aminohexynoate. Distinct changes are seen at three wavelengths. Initially a rapid decrease at 412 nm is accompanied by rapid increases at 330 and 550 nm. Thereafter a slow decrease at both 412 and 550 nm is accompanied by a further rise at 330 nm. The course of these reactions followed at 412 and 550 nm is shown in Fig. 2. The slow process is first order, with the same rate constant that is observed when loss of enzyme activity is followed. This rate constant, measured by activity loss, showed a hyperbolic dependence on inhibitor concentration. The constants were estimated by measuring loss of activity (at 37°C in Tris/acetate pH 8.3, [acetate] = 50 mM) and are given in Table 1.

Both the rate constant (k_F) and the amplitude of the fast change increased with inhibitor concentration. One determination only was made at each of three inhibitor concentrations. The values obtained

Scheme 1. Intermediates in the transamination and inactivation reactions

(a) Reaction between aminohexynoate and any of the transaminases. The horizontal row of formulae represent intermediates in transamination. E_I' is only one of several resonant forms and is better considered as a hybrid with high electron density at 4'-carbon of the coenzyme and at α - and γ -carbons of the inhibitor. Addition of a proton at these positions leads respectively to $E_K I'$ (transamination), $E_L I$ (reversal) or $E_I I$ (inactivation resulting from attack of an enzyme nucleophile at β -carbon of $E_I I$). (b) Likely intermediates in the reactions between aminohexenoate and the enzymes.

at the respective concentrations were: 0.65 mM, $k_F=0.02\text{ s}^{-1}$; 3.25 mM, $k_F=0.05\text{ s}^{-1}$; 6.5 mM, $k_F=0.11\text{ s}^{-1}$.

Fig. 1. Spectral changes associated with the reaction between aminohexynoate and aminobutyrate transaminase

Aminobutyrate transaminase ($7\mu\text{M}$) was made to react with aminohexynoate (0.63 mM) at pH 8.3 and 30°C in a Tris/acetate buffer solution, [acetate]=50 mM. Spectra were recorded before adding inhibitor (highest absorption at 415 nm) and then 5, 12, 20 and 40 min after addition.

We interpret these changes by suggesting that the fast process involves formation of an intermediate with extensive double-bond conjugation due to removal of a proton by the catalytic action of the enzyme (Scheme 1). However, many of the steps of Scheme 1 are not evident and it may be simplified to allow for a number of observations. Firstly exponential loss of activity continues to zero even in the absence of 2-oxoglutarate, which indicates that complete transamination to the free pyridoxamine form of the enzyme (E_m) does not occur extensively at this enzyme concentration. There is an initial rapid increase at 330 nm which occurs at the same rate as the increase at 550 nm and the rapid part of the fall at 412 nm. This may be assigned to an enzyme-inhibitor complex in rapid equilibrium with the

Fig. 2. Course of the reaction between aminohexynoate and aminobutyrate transaminase

Aminobutyrate transaminase ($7\mu\text{M}$) was reacted with aminohexynoic acid (3.3 mM). The course of the reaction was followed continuously at 550 nm (a) and 412 nm (b). Experimental conditions as in Fig. 1.

Table 1. Constants obtained from inactivation experiments

Experimental conditions for each determination are given in the text. The number of determinations of k_{obs} is indicated by n .

Transaminase	Inhibition constants							
	Aminohexynoate				Aminohexenoate			
	$10^3 \times k$ (s^{-1})	K (mM)	k/K ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	n	$10^3 \times k$ (s^{-1})	K (mM)	k/K ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	n
Aminobutyrate	3.8 ± 0.8	1.3 ± 0.2	2.9	11	20.2 ± 2.6	5.1 ± 0.8	3.9	10
Ornithine	10.5 ± 1.4	2.8 ± 0.7	3.8	4	*	*	*	*
Aspartate	9.3 ± 0.7	77 ± 11	0.121	8	†	†	†0.0032	†8

* The reaction between ornithine transaminase and aminohexenoate is very slow and biphasic (see text).

† The reaction between aspartate transaminase and aminohexenoate shows linear rather than hyperbolic dependence on inhibitor concentration. The value entered under k/K is the apparent second-order constant.

550nm-absorbing intermediate, and its 330nm absorption suggests that it may be the enzyme-inhibitor ketimine E_kI' . The limited data available for the last step are consistent with its being a single reversible bimolecular process. This implies that the initial enzyme-inhibitor aldimine E_LI is present only in low concentrations. The simplified scheme may be represented as:

Using the values obtained for the fast rate constant together with the relationships $K = k_{-1}/k_{+1} = 1.3 \text{ mM}$ and $k_F = k_{+1} + k_{-1}$ the values for k_{+1} and k_{-1} were determined to be $12.3 \pm 2.1 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $0.016 \pm 0.003 \text{ s}^{-1}$.

The changes seen when aminobutyrate transaminase was treated with the vinyl analogue, aminohexenoate, were accompanied by a spectral shift from the 412nm-absorbing internal aldimine to a species absorbing at 330nm. In this case the reaction was rapid compared with the time required to scan the spectrum. When followed continuously at 412nm the process appeared first order throughout its course, and whereas with the acetylenic compound changes in concentration of an intermediate absorbing at 550nm were measurable, the increase at higher wavelengths seen with the vinyl analogue was very small. The extent of this change was about 20% of that seen with aminohexynoate and the wavelength of maximum absorbance difference was about 470nm. The absorbance changes were too small to allow the kinetics of changes at this wavelength to be followed.

Loss of activity and decrease in absorbance at 412nm occurred exponentially at the same rate and k_{obs} showed hyperbolic dependence on inhibitor concentration. The kinetic constants were found by measuring loss of activity under the conditions described for aminohexynoate and are given in Table 1.

Although with aminohexenoate the spectral evidence for an intermediate is not as good as for aminohexynoate, it seems probable that in this case a 470nm-absorbing intermediate is formed before inactivation takes place. The lower absorbance at this wavelength is probably due mainly to a lower concentration of the intermediate, which itself is probably due to the higher rate constant for its breakdown into inactive enzyme ($k_{\text{aminohexynoate}} = 0.004 \text{ s}^{-1}$; $k_{\text{aminohexenoate}} = 0.020 \text{ s}^{-1}$). With this inhibitor no significant transamination occurs, since all of the 330nm absorbing material formed is inactive.

Reactions with ornithine transaminase

Ornithine transaminase shares with aminobutyrate transaminase the property of transaminating amino groups from carbon atoms which do not bear a carboxy group. Both aminohexynoate and aminohexenoate react with this enzyme and the results can be fully explained in terms of Scheme 1. Inactivation and transamination occur in both cases, but because the difference in rates of the two processes is greater with the vinyl derivative the two processes can be more clearly separated.

Fig. 3 shows the spectral changes that occur when aminohexenoate reacts with ornithine transaminase. Throughout the period of this experiment and for a further 20min there was no significant loss of activity. Addition of 2-oxoglutarate (5mM) restored the original spectrum, indicating that the original spectral shift was due to transamination of the aldimine form of the enzyme to the amine form. When the enzyme was treated with the same concentration of aminohexenoate, but in the presence of 2-oxoglutarate (5mM), the spectral changes shown in Fig. 4(a) were

Fig. 3. Spectral changes associated with the reaction between aminohexenoate and ornithine transaminase. Ornithine transaminase ($24 \mu\text{M}$) was treated with aminohexenoate ($85 \mu\text{M}$) and absorption spectrum determined at intervals. The spectrum with the highest absorbance at 412nm is that before addition of inhibitor. The other spectra were at 4, 13 and 22min. The reaction was carried out at 30°C in Tris/HCl, pH 8.0, $[\text{Cl}^-] = 10 \text{ mM}$.

seen. This process is much slower than the transamination, gives rise to increased absorbance at 310nm rather than at 330nm, and is accompanied by loss of activity. The enzyme eventually becomes completely inactive and has the absorption spectrum shown in Fig. 4(b). It is clear that with aminohexenoate and ornithine transaminase no detectable inactivation occurs if keto acid is not added because the rate is slow compared with the rate of the protecting transamination reaction. The dependence on inhibitor concentration of the very slow inactivation (which appears slightly biphasic) was not determined. However, in the presence of 1.6mM-inhibitor and 5mM-2-oxoglutarate the slow rate constant was $1.5 \times 10^{-4} \text{ s}^{-1}$ and the transamination rate constant at this inhibitor concentration was $1.8 \times 10^{-2} \text{ s}^{-1}$.

Inactivation of ornithine transaminase by the acetylenic inhibitor aminohexynoate is much faster and is measurable even in the absence of 2-oxoglutarate. For example, when treated with 0.6mM-aminohexynoate the enzyme lost about one quarter of its activity in a non-exponential process lasting a few minutes, but there was no further loss. The

spectral changes seen were almost identical with those of Fig. 3 for aminohexenoate and the 412nm peak was restored to 80% of its original value by adding 2-oxoglutarate. When the reaction was carried out in the presence of 2-oxoglutarate (5mM) the inactivation went to completion in a single first-order process which accompanied decrease in absorbance at 412nm and increase at 330nm. The dependence of this process on inhibitor concentration (determined at 412nm in Tris/HCl, pH8.0, $[\text{Cl}^-]=10\text{mM}$, 30°C) was hyperbolic with $K=2.76 \pm 0.73\text{mM}$ and $k=0.010 \pm 0.001 \text{ s}^{-1}$.

Reactions with aspartate transaminase

Fig. 5 shows the changes seen when these compounds react with aspartate transaminase. The reactions differ in two respects. Firstly, when the acetylenic compound reacts, absorbance at 360nm decreases and is accompanied by simultaneous increases with clear maxima at both 330 and 310nm, whereas the reaction of the vinyl compound results in a new absorbance maximum at 330nm only.

Fig. 4. Effect of 2-oxoglutarate on the reaction between aminohexenoate and ornithine transaminase
(a) Ornithine transaminase ($24 \mu\text{M}$) was treated with 1.6mM-aminohexenoate. The spectrum with highest absorbance at 412nm is that before adding inhibitor, but with 2-oxoglutarate present. Experimental conditions were as in Fig. 3.
(b) This shows the spectrum of the product using a different scale expansion to include the shoulder at 310nm formed as a result of the reaction.

Secondly, although in both cases the spectral changes are accompanied by simultaneous first-order loss of enzyme activity and in both cases k_{obs} increases with inhibitor concentration, the concentration-dependence was linear up to 0.4M with aminohexenoate and hyperbolic with aminohexynoate as inhibitor. The dissociation and rate constants governing the processes (determined at 365nm and at 30°C in Tris/HCl, pH 8.0, $[Cl^-] = 50\text{mM}$) are shown in Table 1.

Addition of 2-oxoglutarate to a final concentration of 2mM did not reverse any of the spectral change, and when this compound was included in the reaction mixture at the start the only effect was to slow the reaction slightly. This decrease in the rate of inhibition may be attributed to the formation of a small amount of abortive complex between the aldimine enzyme and 2-oxoglutarate (Jenkins & D'Ari, 1963).

Comparison of the efficacy of such compounds should take account both of the rapidly reversible part of the reaction and of the irreversible part, and in order to make a numerical comparison the ratio k/K would provide a useful index of specificity.

It is clear that neither of these compounds approaches absolute specificity towards aminobutyrate transaminase. In fact, aminohexynoate is more potent towards ornithine transaminase. However, aminohexenoate is 3 times more effective than aminohexynoate towards aminobutyrate transaminase and its reactivity towards the other enzymes is relatively very slight.

The results obtained with ornithine transaminase show that even at low concentrations of either inhibitor, transamination of the enzyme to E_M goes to completion. This suggests that the keto acid products of the inhibitors are unstable, probably reacting with water. With the other two enzymes release of these products must be slow relative to the irreversible reaction with enzyme. The differences in absorption spectrum of the final inactive product could indicate that different paths to inactivation exist via both EI' and E_KI' . Another explanation could be that the covalent adduct continues to undergo an enzyme-catalysed tautomerization. This would explain why both 310 and 330nm peaks are present in

Fig. 5. Changes in absorption spectrum seen when aspartate transaminase reacts with aminohexynoate and aminohexenoate (a) Aspartate transaminase ($60\mu\text{M}$) was treated with 15mM-aminohexynoate in Tris/HCl buffer, pH=8, $[Cl^-] = 50\text{mM}$. The absorption spectrum with the highest absorbance at 360nm is that of the enzyme before addition of inhibitor and the remaining spectra were recorded after 2, 16 and 30min. (b) Aspartate transaminase ($60\mu\text{M}$) was treated with 22mM-aminohexenoate and absorption spectra were recorded after 23, 46, 72 and 130min. Experimental conditions as in (a).

the inactive enzyme obtained by treating aspartate transaminase with aminohexynoate.

We thank Mr. D. H. Lovell for his expert technical assistance.

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