The reaction of ethanolamine *O*-sulphate with 4-aminobutyrate aminotransferase

Leslie J. FOWLER* and Robert A. JOHN†

*Department of Pharmacology, School of Pharmacy, Brunswick Square, London WC1N 1AX, U.K., and

†Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.

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The enzyme-induced or 'suicide' step by which the substrate analogue ethanolamine *O*-sulphate inactivates 4-aminobutyrate aminotransferase occurs at a rate that is one-tenth that observed for the release of the products of β -elimination, namely ammonia, acetaldehyde and sulphate. An additional reversible reaction not leading to inactivation can be detected spectrally and this decreases the rates of both β -elimination and inactivation. This reaction is ascribed to a step on the normal transamination path, although complete transamination does not occur significantly. The ^{14}C moiety of radiolabelled ethanolamine *O*-sulphate is stably bound to the inactive enzyme in the proportion of 1 mol/mol of active site. The ^{35}S -labelled sulphate moiety is not bound after inactivation, showing that β -elimination must precede inactivation.

Ethanolamine *O*-sulphate (aminoethyl hydrogen sulphate) was shown to be a specific, active-site-directed irreversible inhibitor of partially purified preparations of rat brain 4-aminobutyrate aminotransferase (EC 2.6.1.19) (Fowler & John, 1972). Based on a comparison with the reaction of L-serine *O*-sulphate with aspartate aminotransferase (EC 2.6.1.1) (John & Fasella, 1969) it was reasoned that the compound acted as a pseudo-substrate for the enzyme, which, because of the electronegativity of the sulphate group, was induced to catalyse a β -elimination reaction instead of transamination. Inactivation of the enzyme was believed to be due to the reaction of an abnormal intermediate, perhaps an aminoethylene derivative, with a nucleophile within the enzyme. Such a mechanism gives inhibition that should be specific for two reasons; first, the compound should bind well only to those enzymes whose substrates it resembles strongly, and secondly, the inactivation should occur only in those enzymes that can catalyse the β -elimination. It was found that ethanolamine *O*-sulphate did not inhibit alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase or glutamate decarboxylase (EC 4.1.1.15).

Ethanolamine *O*-sulphate has since proved to be a useful pharmacological tool for increasing the concentration in the brain of the inhibitory neurotransmitter 4-aminobutyrate (Fowler, 1973; Baxter *et al.*, 1973; Anlezark *et al.*, 1976). The principle of enzyme-induced inactivation (often referred to a 'suicide' inactivation) has since become well estab-

lished and inhibitors of this type have been described for several enzymes (for reviews see Seiler *et al.*, 1978). For 4-aminobutyrate aminotransferase the principle has been used in the development of 4-aminohexynoate and 4-aminohexenoate as prospective selective irreversible inhibitors (Jung & Metcalf, 1975; Lippert *et al.*, 1977; John *et al.*, 1979).

The availability of reasonable amounts of purified 4-aminobutyrate aminotransferase from rabbit brain (John & Fowler, 1976) has allowed the reaction with ethanolamine *O*-sulphate to be studied in detail. The present paper is an account of this study and its relation to the mechanism originally proposed.

Materials and methods

Enzyme preparation

A modification of our previously published method (John & Fowler, 1976) gives much better yields and the preparation is more consistently successful. Rabbit brain was stored frozen and thawed when required. A Waring Blendor was used to prepare a 33% (w/v) homogenate in a solution containing sodium acetate (10 mM), EDTA (1 mM), pyridoxal phosphate (0.1 mM), 2-oxoglutarate (1 mM) and 2-mercaptoethanol (0.1%, v/v), and brought to pH 5.4 by adding acetic acid. The homogenate was adjusted to pH 5.3 with 10% (v/v) acetic acid, heated to 53°C for 5 min, cooled to 4°C and centrifuged at 5000 g for 20 min. Centrifugation

was repeated if the solution was not clear. An equal volume of acetone at -20°C was added to the solution at 5°C . After centrifugation the precipitate was dissolved in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] containing 10 mM-sodium acetate and adjusted to pH 7.5. The remaining steps were as described by John & Fowler (1976), except that Hepes replaced Tris in all the buffer solutions. Table 1 gives the details of one such preparation beginning with 1.7 kg of rabbit brain.

Reagents

Pyridoxal 5'-phosphate, 2-oxoglutaric acid, 3,5-diaminobenzoic acid, 4-aminobutyrate and Tris were bought from Sigma (London) Chemical Co., London S.W.6, U.K. Sephadex G-25 was from Pharmacia (G.B.), London W.5, U.K., and Aquasol from New England Nuclear, Boston, MA, U.S.A. Alcohol dehydrogenase (EC 1.1.1.1) was bought from Boehringer (London), Lewes, East Sussex, U.K. All other chemicals were supplied by BDH Chemicals, Poole, Dorset, U.K.

Radiochemicals

Carrier-free $[^{35}\text{S}]$ sulphate (20 mCi) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and, after drying, was taken up in conc. H_2SO_4 (0.2 ml) and used to prepare ethanolamine *O*- $[^{35}\text{S}]$ sulphate by the procedure of Lloyd *et al.* (1961). 2- $[^{14}\text{C}]$ ethanolamine (500 μCi) was obtained from The Radiochemical Centre and reacted with H_2SO_4 in the same procedure in order to prepare 2- $[^{14}\text{C}]$ ethanolamine *O*-sulphate.

Absorption spectra

Absorption spectra and absorption changes were recorded on either a Beckman model 25 or a Cecil CE 505 recording spectrophotometer.

Radioactivity measurements

Aqueous samples (1 ml) of ^{14}C -labelled material were added to Aquasol (10 ml) and their radioactivity was determined in a Nuclear Chicago

Isocap 300 liquid-scintillation counter. The radioactivity of $^{35}\text{SO}_4^{2-}$ on paper cut from electrophoretograms was measured by soaking 1 cm squares in 4 ml of ethoxyethanol for 30 min, and adding 10 ml of a solution (0.5%, w/v) of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole in toluene before scintillation counting. The initial electrophoretic separation was at 200 V for 2 h in 0.2 M-ammonium acetate, pH 4.5.

Ammonia determinations

Ammonia was determined by the Conway (1962) microdiffusion method, using conditions described by Williams & John (1979).

Measurements of enzyme concentration

The concentration of aminobutyrate aminotransferase active sites was measured by titration with the irreversible inhibitor amino-oxyacetate (John *et al.*, 1978). Enzyme activity was measured as described by John & Fowler (1976).

Fitting results and theory

The mechanism described by eqn. (6) (see below) was reduced to that of eqn. (1) by making a number of simplifying assumptions:

Ethanolamine *O*-sulphate was assumed to be constant and saturating throughout on the grounds that its concentration (9.5 mM) greatly exceeded both the total product concentration ($<70\mu\text{M}$) as well as the apparent dissociation constant for initial complex formation ($K_\text{i} = 0.4\text{ mM}$; Fowler & John, 1972). Complex formation was assumed to be relatively very fast. For reasons given in the Results and discussion section elimination of sulphate in the step characterized by k_β of eqn. (6) was considered to be rate limiting for both product formation and inactivation. Thus although the intermediate $\text{E}_\text{L}\text{X}$ of

Table 1. Purification of 4-aminobutyrate aminotransferase from rabbit brain

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol/min per mg}$)	Total activity (units)	Yield (%)
Homogenate	5000	28.3	141 500	0.0124	1750	100
Supernatant after pH 5.3 and 53°C treatment	2800	7.0	19 600	0.0485	950	54.2
Acetone sediment (0–50%)	380	8.8	3344	0.25	836	47.6
DEAE-Cellulose: 10 mM–150 mM-sodium acetate/Hepes (10 mM), pH 7.5 (250 ml each, linear gradient)	250	1.7	425	1.34	569	32.5
CM-Cellulose: 10 mM–110 mM-sodium acetate, pH 5.4 (250 ml each, linear gradient)	140	0.3	42	7.56	317	18.1

eqn. (6) must exist, the system behaves kinetically as though the divergence to product or inactive enzyme occurs directly from $E_L S$. The apparent constants k_p' and k_i' of eqn. (1) are related to the constants of eqn. (6) by:

$$k_p' = \frac{k_p k_\beta}{k_p + k_i} \quad \text{and} \quad k_i' = \frac{k_i k_\beta}{k_p + k_i}$$

Values for the concentrations of $E_L S$, $E_m P'$ and E_i were obtained by using a programmable calculator for numerical integration of the following equations.

$$-\delta[E_L S] = (k_{+1} + k_i')[E_L S] - k_{-1}[E_m P']\delta t \quad (2)$$

$$\delta[E_m P] = k_{+1}[E_L S] - k_{-1}[E_m P']\delta t \quad (3)$$

$$\delta[E_i] = k_i'[E_L S]\delta t \quad (4)$$

$$\delta[P] = k_p'[E_L S]\delta t \quad (5)$$

The time interval δt chosen for each iteration was 100-fold smaller than the reciprocal of the fastest rate constant and adjustments to the rate constants were made until the fit to the experimental points was judged subjectively to be adequate.

Results and discussion

Spectral changes and the course of inactivation

The bound coenzyme of 4-aminobutyrate aminotransferase has an absorbance spectrum with peaks at 415 nm and 330 nm. Addition of ethanolamine sulphate produced a decrease in absorbance at 415 nm and a simultaneous increase at 330 nm (Fig. 1). Assay of samples withdrawn from the solution showed that enzyme activity was lost progressively. The course of each of these reactions is shown in Fig. 2. Analysis of these and other similar results showed that although the reaction producing the spectral changes could reasonably be described as first order ($k_{\text{obs.}} = 5.3 \text{ min}^{-1}$), the course of inactivation was clearly slower and not first order.

Reaction with [^{14}C]ethanolamine sulphate

The enzyme was inactivated with [^{14}C]ethanolamine sulphate at room temperature. A portion of the inactivated enzyme was separated on a Sephadex G-25 column and enzyme activity, absorbance at 330 nm and radioactivity of each 1 ml fraction were determined. Enzyme activity was zero throughout and a peak of radioactivity coincided with the enzyme eluting in the void volume (Fig. 3). The specific radioactivity of the separated labelled enzyme ($138 \pm 8 \text{ c.p.m./nmol}$) was the same as that of the ethanolamine sulphate used ($135 \pm 8 \text{ c.p.m./nmol}$). We conclude therefore that 1 mol of that part of the substrate containing C-2 is bound irreversibly per mol of active site.

Fig. 1. Spectral changes occurring when 4-aminobutyrate aminotransferase reacts with ethanolamine *O*-sulphate

The enzyme (0.375 ml; $30 \mu\text{M}$) in 15 mM-acetic acid, adjusted at room temperature to pH 8.2 with solid Tris, was treated with $25 \mu\text{l}$ of ethanolamine *O*-sulphate to give a final concentration of 8.9 mM and incubated for 1 h at 37°C . The spectrum with the higher peak at 415 nm and lower at 330 nm is that of the isolated enzyme and the other is that recorded after reaction with ethanolamine *O*-sulphate.

Reaction with ethanolamine [^{35}S]sulphate

The enzyme (0.8 ml, $8 \mu\text{M}$) was inactivated with ethanolamine [^{35}S]sulphate (9.5 mM, 5.6 Ci/mol) for 1 h and under the same conditions as in the experiment with the ^{14}C -labelled compound. The inactive enzyme was chromatographed on Sephadex G-25 as before. A small amount of radioactivity was eluted in the void volume with the protein peak but this corresponded to only 0.05 mol of $^{35}\text{SO}_4^{2-}$ /mol of active site. Sulphate must therefore be eliminated before inactivation takes place.

Samples ($5 \mu\text{l}$) were taken during the course of inactivation and analysed for free inorganic $^{35}\text{SO}_4^{2-}$ after electrophoretic separation. Although the measurements made by this method were not very precise it was clear from comparing the course of sulphate elimination with that of inactivation, that, in molar terms, the amount of sulphate released was always an order of magnitude greater than the enzyme inactivated. In two separate experiments when 6 nmol of enzyme ($8 \mu\text{M}$) was treated with

Fig. 2. Course of spectral changes and inactivation

The enzyme (0.75 ml; $9.3 \mu\text{M}$) in Tris/acetate buffer, pH 8.2, was treated with $50 \mu\text{l}$ of ethanolamine *O*-sulphate to give a final concentration of 8.9 mM. The reaction was followed at 330 nm (a) and at 415 nm (b). In an identical experiment samples ($5 \mu\text{l}$) were withdrawn and assayed for enzyme activity (c).

Fig. 3. Gel filtration of 4-aminobutyrate aminotransferase after inactivation with $[2\text{-}^{14}\text{C}]$ ethanolamine *O*-sulphate

The enzyme ($41 \mu\text{M}$) in 1.9 ml of 15 mM-Tris/acetate buffer, pH 8.2, was treated with $[2\text{-}^{14}\text{C}]$ ethanolamine sulphate (9.5 mM) for 16 h at room temperature. A portion (1.5 ml) was applied to a column ($55 \text{ cm} \times 1.5 \text{ cm}$) equilibrated with 5 mM-acetic acid that had been adjusted to pH 8.2 with solid Tris. Fractions (1 ml) eluted were analysed for A_{330} (○) and radioactivity (●). Enzyme activity was zero throughout.

9.5 mM-ethanolamine *O*-sulphate 66 nmol and 62 nmol of inorganic sulphate were released when enzyme inactivation was complete.

Formation of ammonia

Elimination of sulphate from ethanolamine *O*-sulphate yields aminoethylene, which would be expected to break down to ammonia and acetaldehyde after tautomerization to the appropriate imine. The amount of ammonia released was measured after treating 23 nmol of enzyme in 0.8 ml of Tris/acetate buffer, pH 8.2, with 9.5 mM-ethanolamine sulphate at 37°C for 1 h. After subtracting small control values in experiments from which either the compound or the enzyme was omitted, 253 nmol of ammonia was measured, indicating a ratio of product formation to enzyme inactivation of 11.

Comparison of the course of elimination, inactivation and spectral change

The methods for measuring sulphate and ammonia were inconvenient for continuous, accurate determination of product concentration. However, acetaldehyde can be conveniently measured by using NADH and alcohol dehydrogenase, so long as the spectral transitions of the aminotransferase itself are eliminated. NADH oxidation was therefore measured at the isosbestic point for the amino-

Fig. 4. Reactions of ethanolamine sulphate with aminobutyrate aminotransferase

The enzyme (0.9 ml, 5.2 μM) in 15 mM-acetic acid adjusted to pH 8.2 with solid Tris was reacted with ethanolamine sulphate (9.2 mM) at 37°C. Acetaldehyde production was followed continuously using yeast alcohol dehydrogenase (5 units/ml) and NADH (0.13 mM). The points (●) are taken from a continuous recording of A_{362} . Enzyme inactivation (▲) was determined by removing 5 μl samples at the intervals. The concentration of E_i and E_mP (■) are determined from data similar to those of Fig. 2 using an absorption coefficient for the conversion of ES into either species of 3300 $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The continuous lines for all three curves are theoretical and derived from eqn. (6) using the rate constants $k_{+1} = 0.2 \text{ min}^{-1}$, $k_{-1} = 0.2 \text{ min}^{-1}$, $k_\beta = 4.6 \text{ min}^{-1}$ and $k_p/k_i = 12.5$. The method used to obtain the fit is described in the Materials and methods section.

transferase spectral changes (362 nm) and calculations were made using $\epsilon_{362}^{\text{NADH}} = 4300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Fig. 4 shows the course of acetaldehyde production and also shows the progress of both the spectral transition at 415 nm and inactivation for comparison. Acetaldehyde production and inactivation are both biphasic showing an initial 'burst'. This suggests the transient formation of a species off the common pathway that leads both to inactivation and product formation. We propose that this species (E_mP' in eqn. 6) has an absorbance spectrum similar to that of the final inactive enzyme and that its formation during the early part of the reaction accounts in part for the rapid spectral transition. Eqn. (6) is intended to summarize the reactions:

Fig. 5. Structures proposed for the intermediates of eqn. 1. Substituents in the C-2 and C-5 positions have been omitted for clarity.

E_L is the free pyridoxalimine form of the enzyme. $E_L S$ represents at least two intermediates in rapid equilibrium, namely the imine between enzyme and substrate and the quinonoid structure formed after loss of the α -proton. Elimination of the β -sulphate leads to $E_L X$, which is suggested to be an imine of aminoethylene and the coenzyme (Fig. 5). This is either released to give products or it leads to inactivation by an internal reaction with an enzyme nucleophile. The reversible formation of $E_m P$, which is fully active in the normal enzyme assay, could very reasonably be loss of the proton followed by reprotonation at C-4' of the coenzyme. This step would lead to complete transamination if $E_m P'$ dissociated to E_m and P' . We deduce that $E_m P$ does not dissociate extensively because if most of the spectral change were due to conversion into free E_m then addition of 2-oxoglutarate would effectively reverse this transamination and maintain rapid inactivation and β -elimination. In fact in an experiment using 20 mM-2-oxoglutarate we found the rates to be decreased, presumably because of competition with the ethanolamine sulphate. This observation is in complete contrast with the behaviour of the analogous aspartate aminotransferase/serine *O*-sulphate system (John & Fasella, 1969) in which 2-oxoglutarate accelerates both processes very markedly. In this latter system $E_m P$ dissociates rapidly and extensively to the free pyridoxamine form of the enzyme, thus virtually stopping both β -elimination and inactivation under some conditions.

Eqn. (6) was used to predict the course of β -elimination and inactivation. Ethanolamine *O*-sulphate was assumed to be effectively saturating and unchanged throughout and values for $[P]$, $[E_i]$ and the sum $[E_m P] + [E_i]$ were predicted by numerical integration of the appropriate differential equations. The rate-limiting step on the path to inactivation and product formation was ascribed to the β -elimination step characterized by k_β . This was because $E_L X$ would be expected to absorb at high wavelength as it has more extensive conjugation than the other species and no intermediate with these spectral characteristics accumulates significantly. The best fit for all three processes with the experimental values is shown in Fig. 4, which also gives the rate constants used to obtain the fit.

It may seem curious that both active and inactive enzymes have spectra with peaks in the same

positions and that the only difference is the relative sizes of the peaks. However, it is unlikely that the nature of the bonding of the coenzyme to the enzyme is greatly altered by the inactivation. If, as for aspartate aminotransferase (Morino & Okamoto, 1973; John *et al.*, 1973), the enzyme nucleophile involved is the lysine that normally binds the coenzyme, then the inactivation simply involves a small increase in the length of the lysine side chain.

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