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The reactions of aminobutyrate aminotransferase and ornithine aminotransferase with analogues of ethanolamine *O*-sulphate

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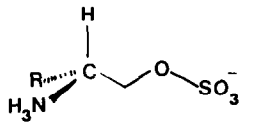
Ethanolamine *O*-sulphate (2-aminoethyl sulphate) is an effective 'suicide' (enzyme-activated irreversible) inhibitor of 4-aminobutyrate aminotransferase (EC 2.6.1.19) [1]. By inactivating the brain enzyme *in vivo* it raises the concentration in brain of the inhibitory neurotransmitter that is the enzyme's substrate. As a result, treated animals are protected against convulsions [2–4]. Ethanolamine sulphate is very polar and would, thus, be expected to penetrate poorly to the target enzyme in brain cells. Oral administration of the compound in drinking water requires large doses and the degree of inhibition of the enzyme and consequent increases in aminobutyrate concentration are not so large as those produced by direct intracerebroventricular injection. We, therefore, wished to prepare analogues of the compound having decreased polarity but retaining the structural elements that make it an effective 'suicide' inhibitor. The inactivation mechanism requires that several features of the parent molecule be retained. The sulphate group performs two functions in mimicking the carboxyl group of aminobutyrate by furnishing an appropriately placed negative charge for binding, and in providing a good leaving group whose elimination generates the inactivating electrophile at the enzyme's active site. The amino group is essential for imine formation with the enzyme's coenzyme pyridoxal phosphate. Of the two protons on the 4-carbon of aminobutyrate only one, the pro-*S*, is labilized in the critical catalytic step [6, 7]. The inhibitory mechanism requires that the corresponding hydrogen in a suicide inhibitor be retained. The pro-*R* 4-hydrogen of aminobutyrate is not labilized and the enzyme's effective use of L-glutamate as substrate shows that bulky groups can be accommodated in this position [8]. Furthermore, compounds with acetylenic and vinyl groups in this position are good inhibitors of the enzyme [9, 10]. Thus, we chose to synthesize analogues of ethanolamine *O*-sulphate substituting the pro-*R* hydrogen with hydrophobic groups (Fig. 1).

The compounds D-alaninol *O*-sulphate (R = CH_3) and D-methioninol *O*-sulphate (R = $-CH_2-CH_2-S-CH_3$) fulfil these requirements and the present paper describes their synthesis by reduction of the appropriate D-amino acid and sulphation of the resulting alcohol. The reactions of these compounds with aminobutyrate aminotransferase are described. As a test of specificity, the reactions of these compounds with ornithine aminotransferase (EC 2.6.1.13) were also examined on the grounds that this enzyme cat-



R = H– ethanolamine sulphate
R = CH_3– alaninol sulphate
R = CH_3–S–$(CH_2)_2$– methioninol sulphate

Fig. 1. Structure of ethanolamine sulphate analogues.

alyses a closely-related and stereochemically identical reaction on ornithine and is known to be inactivated by other compounds intended as 'suicide' inhibitors of aminobutyrate transaminase [7, 9].

Methods

Aminobutyrate aminotransferase was purified to homogeneity from rabbit brain as described earlier [12]. Ornithine aminotransferase was purified to homogeneity from rat liver using a modification [7] of the method of Peraino *et al.* [13]. D-Alanine, D-methionine ethyl ester and L-alaninol were bought from Sigma, London, U.K. 2-oxoglutarate from Koch-Light Laboratories, Colnbrook, U.K. and $^{35}SO_4$ from Amersham International Amersham, U.K. Other chemicals were from BDH, Poole, U.K.

Aminobutyrate aminotransferase activity was assayed by the method of Salvador and Albers [14] and ornithine aminotransferase by the method of Peraino and Pitot [15]. Molar concentrations of the pure enzymes were determined from the absorbance of bound coenzyme as described earlier [9]. Reactions of the compounds with both enzymes were carried out in 10 mM Hepes adjusted to pH 8.0 with sodium hydroxide. Absorption spectra during these reactions were determined on a Beckman Model 25 recording spectrophotometer. $^{35}SO_4$ was determined by paper electrophoresis at 8000 V for 10 min in 0.16 M acetic acid. After drying, 1 cm squares of electrophoresis paper were counted in 18 ml of Fisofluor 1 in an Intertechnique 4000 liquid scintillation counter.

Syntheses

Finely divided D-alanine (5 g) was added slowly to 2.8 g of lithium aluminium hydride in 100 ml of ice-cold tetrahydrofuran. After the initial vigorous reaction, the mixture was allowed to rise to room temperature and maintained for 24 hr. Excess lithium aluminium hydride was destroyed by adding 50 ml of ethyl acetate. Saturated aqueous sodium sulphate was then added until a densely packed precipitate formed. The clear supernatant was decanted, neutralized with 1 M sulphuric acid and dried by rotary evaporation. The residue was dissolved in 3 ml water and applied to a column of Dowex 1 (10 × 1.4 cm, OH^- form, 8% cross-linked equilibrated with water). Aqueous alaninol was extracted by distillation with all material distilling up to 220° being collected. The colourless distillate was neutralized with sulphuric acid, evaporated to dryness and the white solid recrystallized from hot wet ethanol (yield = 300 mg). The compound chromatographed identically with authentic L-alaninol and gave the same characteristic colour with ninhydrin. Its NMR spectrum was also identical with that of L-alaninol.

The D-alaninol was converted into its *O*-sulphate ester by the method of Lloyd *et al.* [16]. The white crystalline solid (280 mg) had the predicted NMR spectrum and chromatographed as a single spot on TLC in butanol–acetic acid–water, 3:1:1. Carbon, hydrogen and nitrogen analysis gave: C, 24.3%; H, 6.2%; and N, 8.5%; theory: C, 23.2%, H, 5.8%; and N, 9.0%.

D-Methioninol *O*-sulphate was prepared by the same method except that D-methionine methyl ester was used as starting material. The alcohol formed in the reduction step was extracted by recrystallization from hot wet ethanol without prior distillation. The white crystalline solid had the predicted NMR spectrum and chromatographed as a single spot on TLC in butanol–acetic acid–water, 3:1:1. Carbon, hydrogen and nitrogen analysis gave: C, 28.9%; H, 6.6%; and N, 6.3%; theory: C, 27.9%, H, 6.1% and N, 6.5%.

Radiolabelled D-alaninol *O*-[^{35}S]sulphate was prepared from 300 mg of D-alaninol using 20 mCi of sodium [^{35}S]sulphate in 1 ml conc. H_2SO_4, again using the method of Lloyd *et al.* [16]. Immediately before using the labelled compound as a substrate the small amounts of inorganic $^{35}SO_4$ that formed spontaneously were removed by adding 1 g of unlabelled sodium sulphate to 5 ml of D-alaninol [^{35}S]sulphate (40 mg/ml). Dry methanol was added to precipitate sulphate and after centrifugation the clear solution was evaporated to dryness over sodium hydroxide.

Results

Figure 2 shows the spectral changes that take place over about 80 min when 20 mM D-alaninol sulphate was reacted with 13 μM aminobutyrate aminotransferase. Absorbance decreased at 412 nm and increased at 330 nm. However, the changes at the two wavelengths did not exactly parallel each other as most of the increase at 330 nm occurred within 4 min, whereas the decrease at 412 nm continued for about 20 times longer. When, in separate experiments, the D-alaninol sulphate was used at lower concentrations (down to 5 mM) the spectral changes at each wavelength came to parallel each other more closely because, although both could be seen to become slower, the effect on the rapid phase was more pronounced. Semi-logarithmic analysis of the data at 412 nm showed two first-order reactions the fastest of which increased to a limiting value as alaninol sulphate concentration was increased. The data were not adequate to analyse the slow phase.

When ornithine aminotransferase (32 μM) was treated with D-alaninol sulphate (10 mM), the spectral changes shown in Fig. 2(b) were seen. Semi-logarithmic plots of A_{420} against time were linear and the observed rate constants increased linearly over the concentration range examined. When the normal keto acid substrate, 2-oxoglutarate (0.6 mM) was added to either enzyme after the spectral transition was complete the original spectrum was largely restored.

When aminobutyrate aminotransferase (13 μM) was treated with D-methioninol sulphate (10 mM) no spectral changes occurred in 4 hr. However, with ornithine transaminase slow reactions were seen giving the same spectral changes seen with D-alaninol sulphate. The half-time ($t_{\frac{1}{2}}$) for this change was 240 min.

When aminobutyrate aminotransferase (26 μM) was treated with D-alaninol sulphate (25 mM), samples removed at intervals and measured for enzyme activity, a very slow inactivation was seen. After 5 hr inactivation was 18% and after 24 hr 45%. During this time a control, from which D-alaninol sulphate was omitted, lost no activity. Inclusion of 2-oxoglutarate (2.4 mM) prevented any loss of activity from the D-alaninol sulphate treated sample.

When ornithine aminotransferase (36 μM) was treated with D-alaninol sulphate (25 mM), activity was lost in a slow exponential process ($t_{\frac{1}{2}}$ = 32 hr). Again, 2-oxoglutarate (5.2 mM) prevented loss of activity. Aminobutyrate aminotransferase was not inactivated by D-methioninol-sulphate but this compound (25 mM) inactivated ornithine aminotransferase exponentially but very slowly ($t_{\frac{1}{2}}$ = 24 hr).

Aminobutyrate aminotransferase (10 μM) was treated with D-alaninol [^{35}S]sulphate (20 mM, 1.1 Ci/mole) and 10 μl samples were removed at intervals over 3.5 hr. The samples were assayed for $^{35}SO_4$ electrophoretically. There

was no production of $^{35}SO_4$ during the experiment, although an amount equimolar with the enzyme would readily have been detected. A radioactive compound having electrophoretic mobility about half that of $^{35}SO_4$ appeared within 20 min and did not increase further. When 2-oxoglutarate (5 mM) was included in the reaction mixture the amount of this compound formed increased threefold.

Discussion

It is clear that neither of these compounds is a 'suicide' inhibitor of either enzyme. However, it may be of value to ascertain why these homologues of the potent inactivator ethanolamine sulphate are ineffective.

The spectral transition observed with D-alaninol sulphate and aminobutyrate aminotransferase is similar in most respects to that seen when ethanolamine *O*-sulphate inactivates the enzyme. The changes occur at the same wavelengths and, in both cases, biphasic processes of similar time scale occur [12]. However, the spectral change brought about by the alaninol derivative is reversed by 2-oxoglutarate, whereas that occurring with ethanolamine sulphate is not. This is a major difference indicating separate identities for the 330 nm absorbing forms of the coenzyme produced by the different compounds. The reversal produced by the normal keto acid substrate, 2-oxoglutarate, shows that the spectral change produced by alaninol sulphate is, very probably, due to transamination of the coenzyme into the pyridoxamine form and not to inactivation as seen with ethanolamine sulphate. Thus, alaninol sulphate acts as a poor transamination substrate for aminobutyrate aminotransferase and ornithine aminotransferase. With aminobutyrate aminotransferase the rate constant for the rapid spectral change rose to a limiting value as alaninol sulphate concentrations were increased. We interpret this to indicate saturation and, using the method of Wilkinson [17] we have determined the Michaelis constant (K_m) and the rate constant (k) for the process to be 8.2 ± 3.5 mM and 2.6 ± 0.6 min^{-1}, respectively. No saturation was observed with ornithine aminotransferase, a linear dependence of observed first-order rate constant on alaninol sulphate concentration being found up to 25 mM. In this case the reaction may be interpreted as a simple bimolecular process characterized by a second-order rate constant of 6.3 $mM^{-1}\,min^{-1}$.

Transamination requires enzyme catalysed labilization of the proton on C-4 of the substrate. The spectral changes show clearly that alaninol sulphate binds to the enzyme and that the relevant proton is labilized. The K_m for the reaction is about 10 times higher than the analogous constant for the multiple reactions occurring between this enzyme and ethanolamine sulphate. If proton abstraction is the rate limiting step in the transamination process, then it is very much slower ($k = 0.18\,sec^{-1}$) than in the case of ethanolamine sulphate (4.6 sec^{-1}).

The enzyme catalysed elimination of the C-4 proton results in a carbanion–quinonoid intermediate (Fig. 3) the

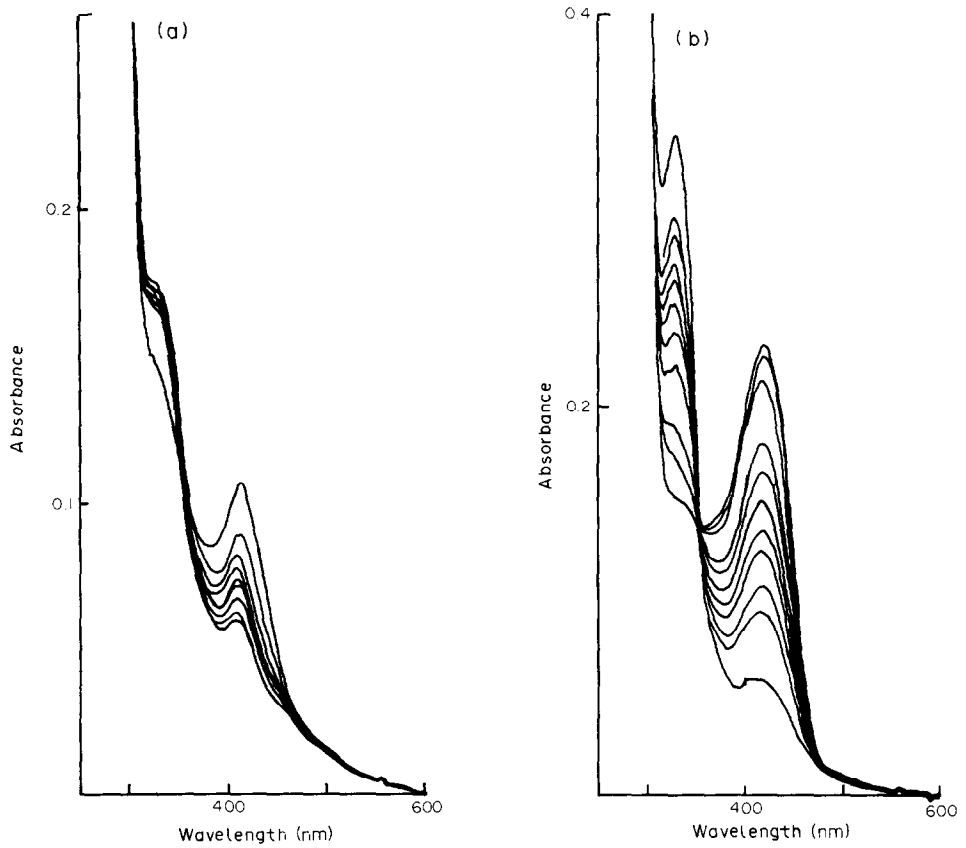


Fig. 2. Spectral changes seen when aminobutyrate aminotransferase and ornithine aminotransferase were reacted with D-alaninol sulphate. (a) Aminobutyrate aminotransferase (13 μM) was treated with D-alaninol sulphate (20 mM). Spectra were recorded at 2, 7, 11, 40, 96, 126 and 196 min after mixing. (b) Ornithine aminotransferase (32 μM) was treated with D-alaninol sulphate (10 mM). Spectra were recorded at 2, 14, 24, 49, 71, 97, 106, 170, 215 and 1200 min after mixing. The reactions with both enzymes were carried out in 10 mM Hepes that had been adjusted to pH 8.0 with sodium hydroxide and at 30°. In both cases the spectra with highest absorbance at 415 nm is that of the untreated enzyme.

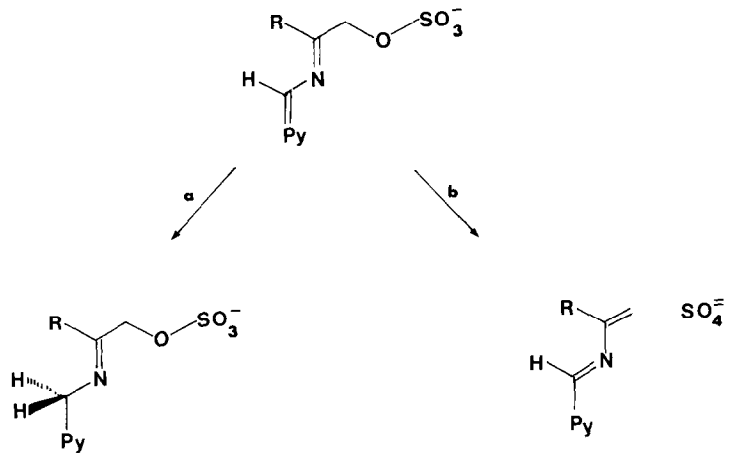


Fig. 3. Structure and fate of the carbanion–quinonoid intermediate. The carbanion–quinonoid intermediate has two fates. (a) It may be protonated at the 4′-position of the coenzyme giving a saturated tetrahedral carbon. (b) It may eliminate sulphate. When R is an alkyl group the planar geometry of the carbanion–quinonoid intermediate is sterically hindered. This hindrance is relieved by (a) but remains in (b). Thus hindrance, which is a consequence of (a), is favoured over elimination.

extra negative charge of which may be lost either by protonation at C-4′ of the coenzyme, in a process leading to transamination (a) or by elimination of sulphate together with the extra electrons (b). Thus, the fate of the quinonoid intermediate determines the outcome of the overall reaction. The effect of an alkyl group, R, in the place of hydrogen is firstly, to slow down formation of this intermediate because the steric interaction between R and the imine hydrogen inhibits formation of the intermediate which is constrained by conjugation to be approximately planar. Process (a) (Fig. 3) then occurs relatively rapidly as the molecular strain is reduced in the less rigid product. On the other hand, process (b) is not so favoured because the strain remains.

The slow inactivations occurring when the enzymes were treated with these analogues are different from the 'suicide' process seen with ethanolamine sulphate in that they are prevented by 2-oxoglutarate. We conclude that the inactivations reported in the present paper are consequences of transamination. In the absence of oxoglutarate transamination yields enzyme with the coenzyme in the weakly bound pyridoxamine phosphate form and it is likely that the inactivation observed occurs through spontaneous denaturation of the apoenzyme formed when the coenzyme dissociates. When D-alaninol [^{35}S]sulphate was used as substrate an acidic product, having lower electrophoretic mobility than $^{35}SO_4$, was found. The amount of this product increased when 2-oxoglutarate was included at the beginning of the reaction. This compound is probably the keto acid formed by transamination of alaninol sulphate.

It is, thus, clear that alaninol sulphate and methioninol sulphate (Fig. 1) are substrates for aminobutyrate transaminase and ornithine transaminase. Although they are close analogues of ethanolamine sulphate, they differ from it in lacking its powerful enzyme-activated irreversible inhibition. This difference is attributed to a failure by the analogues to eliminate sulphate after the initial loss of H^+ catalysed by the enzymes.

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