Stereochemistry of the reaction of the inhibitor β-chloroalanine with mercaptoethanol, a β-substitution reaction catalysed by an aminotransferase

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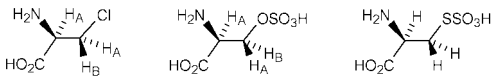
L-Aspartate aminotransferase, a member of the α-family of PLP mediated enzymes, which normally catalyses transamination, has been used to catalyse the β-substitution reaction of stereospecifically labelled samples of the enzyme inhibitor β-chloro-L-alanine with 2-mercaptoethanol; the stereochemistry of the products was assigned by independent synthesis, showing that the abnormal substitution reaction proceeds with overall retention of stereochemistry, the usual stereochemical consequence of reactions catalysed by enzymes of the β-family of PLP mediated enzymes which have low homology with enzymes of the α-family.

Pyridoxal phosphate (PLP) dependent enzymes catalyse a wide variety of different reactions, mainly involving amino acids. Early theories[1–3] to generalise and explain the chemical basis for these widely differing reactions have largely been confirmed. Dunathan and Voet[4] proposed that PLP-dependent enzymes evolved from a common ancestor protein, and there is evidence[5] that this is true for one of the three families of PLP mediated enzymes, the α-family, which contains a predominant number of enzymes. Homology studies have recently suggested that the α- and γ-families of enzymes may be distantly related but that enzymes of the β-family are not closely related to either of these families.[5]

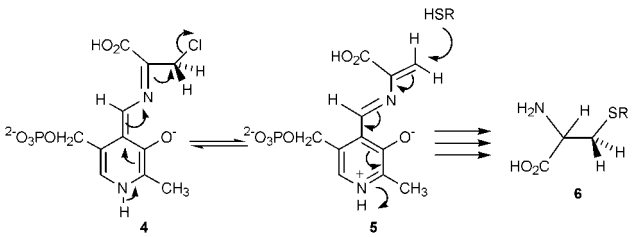
L-Aspartate aminotransferase (EC 2.6.1.1) is an enzyme of the α-family, catalysing the transamination of L-aspartic acid to α-ketoglutaric acid giving oxaloacetic and glutamic acids. β-Chloro-L-alanine **1**[6,7] has been shown not only to inhibit this enzyme, but also to be converted to pyruvic acid by it. It has also been observed that introduction of thiosulfate during the inactivation of L-aspartate aminotransferase by L-serine-*O*-sulfate **2** reversed inhibition and caused production of L-cysteine-*S*-sulfonate **3**.[8] Further, inhibition of D-amino acid



1 **2** **3**

aminotransferase by β-bromo-D-alanine was reversed by addition of thiols and, when β-mercaptoethylamine was used, *S*-(β-aminoethyl)-D-cysteine and Δ^1-thiomorpholine-2-carboxylic acid were products.[9] Thus these enzymes have catalysed β-substitution reactions typical of enzymes of the β-family, rather than transamination which is typical of α-family enzymes. This would suggest that the quinonoid form **4**, formed in the 'normal' reaction, instead of protonation at C-4′ by Lys258, converted to **5** in the inhibition reaction. This may be processed to pyruvate or an inhibitor complex in the absence of a thiol. In the presence of a thiol, however, the reaction presumably proceeds as shown in Scheme 1 eventually to give the final cysteine derivative **6**.

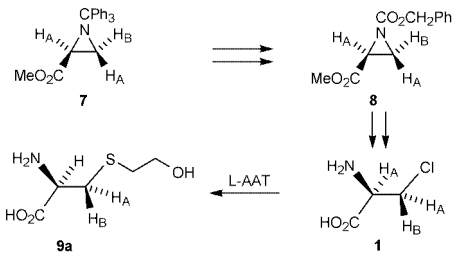
The change in role from catalyst for transamination to catalyst for β-substitution exhibited by aspartate aminotransferase on change of substrate from L-aspartate to β-chloro-L-alanine **1** was intriguing and we decided to examine the overall stereochemistry of the β-substitution reaction in the presence of thiol to see whether it was in keeping with the retention generally shown by reactions catalysed by the enzymes whose



4 **5** **6**

Scheme 1

normal role this was.[10] We therefore prepared samples of β-chloro-L-alanine **1** which were stereospecifically labelled at C-3. This was achieved by first converting the labelled samples of methyl (2*S*)-*N*-tritylaziridine-2-carboxylate **7**(H_B = ^2H)[11] and **7**(H_A = ^2H)[11] to the corresponding urethanes **8**† by reaction with trifluoroacetic acid in chloroform to remove the trityl group, followed immediately by reaction with benzyl chloroformate under Schotten Baumann conditions (Scheme 2). β-Substitution with chloride ion was achieved by reaction with $TiCl_4$ in CH_2Cl_2–$CHCl_3$, and deprotection in refluxing 4 M H_2SO_4 then gave the enzyme inhibitors **1**.† The ^1H and ^2H NMR spectra indicated that the inhibitors **1** were unique diastereoisomers and, since substitution is accompanied by inversion of stereochemistry at the labelled β-position, these were assigned as (2*R*,3*S*)-[3-$^2H_1$]- and (2*R*,3*R*)-[2,3-$^2H_2$]-β-chloroalanine, **1**(H_B = ^2H) and **1**(H_A = ^2H), respectively.



7 **8** **9a** **1**

Scheme 2

The enzyme L-aspartate aminotransferase (EC 2.6.1.1) was isolated by a standard purification protocol[12] from *E. coli* TY103[13] which was transformed with plasmid pKDHE19/AspC.[14] It had specific activity of 36.57 units mg^{-1} and appeared as one major band of molecular weight 43000 Da on SDS-PAGE. Incubation with (2*R*,3*R*)-[2,3-$^2H_2$]-β-chloroalanine **1**(H_A = ^2H) in the presence of a variety of thiols and under various conditions was now undertaken. The best results were obtained at pH 8.4 when mercaptoethanol was employed‡ and samples of labelled 3-(2-hydroxyethyl)cysteine **9a** were obtained in which the α-deuterium atom had exchanged as expected from the mechanism in Scheme 1. The β-deuterium atom also exchanged on prolonged incubation, an effect which had been previously noted in reactions with this enzyme.[15,16] However, when the incubation was stopped after 3 h, a sample of (2*R*)-3-(2-hydroxyethyl)cysteine **9a** was obtained with a ^1H NMR spectrum which showed that there was stereospecific

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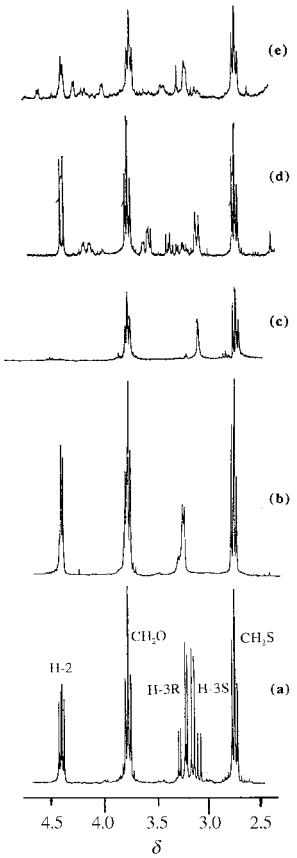
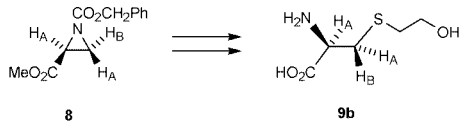


Fig. 1 ^{1}H NMR spectra in 10% ^{2}HCl–^2H_2O of (a) synthetic (2*R*)-3-(2-hydroxyethyl)cysteine **9**, (b) synthetic (2*R*,3*S*)-[3-$^2H_1$]-3-(2-hydroxyethyl)cysteine **9b**(H_B = ^{2}H), (c) synthetic (2*R*,3*R*)-[2,3-$^2H_2$]-3-(2-hydroxyethyl)cysteine **9b**(H_A = ^{2}H), (d) product from incubation of (2*R*,3*R*)-[2,3-$^2H_2$]-β-chloroalanine **1**(H_A = ^{2}H) with L-aspartate aminotransferase and (e) product from incubation of (2*R*,3*S*)-[3-$^2H_1$]-β-chloroalanine **1**(H_B = ^{2}H) with L-aspartate aminotransferase.

labelling at C-3 [Fig. 1(d)]. (2*R*,3*S*)-[3-$^2H_1$]-3-β-Chloroalanine **1**(H_B = ^{2}H) was therefore incubated for the same time when the ^{1}H NMR spectrum [Fig. 1(e)] of the product **9a** indicated that it was the C-3 epimer.

The trapping reaction was evidently stereospecific and it was now necessary to determine the absolute stereochemistry of the overall reaction. This was achieved by independent synthesis of samples of (2*R*)-3-(2-hydroxyethyl)cysteine **9b** which were labelled stereospecifically with deuterium at C-3 in an unambiguous manner. This is shown in Scheme 3, the labelled carbobenzyloxyaziridines **8** being reacted with mercaptoethanol containing a catalytic quantity of boron trifluoride etherate. Inversion of stereochemistry at the labelled atom, C-3, is expected and ^{1}H and ^{2}H NMR spectra showed that the labelled products† were single diastereoisomers. Hydrolysis in refluxing 4 M H_2SO_4 then gave the free amino acids **9b**.



8 **9b**

Scheme 3

The ^{1}H NMR spectra of the synthetic samples of (2*R*,3*S*)-[3-$^2H_1$]-3-(2-hydroxyethyl)cysteine **9b**(H_B = ^{2}H)† [Fig. 1(b)] and (2*R*,3*R*)-[2,3-$^2H_2$]-3-(2-hydroxyethyl)cysteine **9b**(H_A = ^{2}H)† [Fig. 1(c)] allowed the 3-*pro-S* and 3-*pro-R* protons in the spectrum of 3-(2-hydroxyethyl)cysteine **9** to be assigned and therefore the absolute stereochemistry of the incubation products to be deduced. It was evident that the product from the incubation using (2*R*,3*S*)-[3-$^2H_1$]-3-β-chloroalanine **1**(H_B = ^{2}H) was (2*R*,3*S*)-[3-$^2H_1$]-3-(2-hydroxyethyl)cysteine **9a**(H_B = ^{2}H) [Fig. 1(e)] and that the product when (2*R*,3*R*)-[2,3-$^2H_2$]-3-β-chloroalanine **1**(H_B = ^{2}H) was used was (2*R*,3*R*)-[3-$^2H_1$]-3-(2-hydroxyethyl)cysteine **9a**(H_A = ^{2}H) [Fig. 1(e)].

These results imply that the β-replacement reaction, which in this case is catalysed by an enzyme of the α-family whose normal function is transamination, occurs with overall retention of stereochemistry. This is the general expectation[10] for the PLP-mediated enzymes of the β-family where β-replacement reactions are the norm and may imply a closer relationship between families than homology[5] suggests. Although X-ray structures are available for aspartate aminotransferase[17] and other α-family enzymes, tryptophan synthase (EC 4.2.1.20) is one of the few enzymes of the β-family whose tertiary structure has been defined by X-ray cystallography.[18] It is interesting that, in the presence of thiols, tryptophan synthase has been shown to catalyse transamination and β-replacement of L-serine by mercaptoethanol.[19] Further, the Lys87Thr mutant of tryptophan synthase will not turn over the natural substrate serine in the absence of NH_4^+ but it will turn over β-chloro-L-alanine.[20]

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Notes and references

† These compounds had the expected analytical and spectroscopic properties.

‡ Incubation conditions: β-chloro-L-alanine: (0.08 mmol) and 2-mercaptoethanol (0.15 mmol) in sodium arsenate buffer (2 ml) containing 80 units of enzyme.

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