Stereochemical Studies of Processing of D- and L-isomers of Suicide Substrates by an Amino Acid Racemase from *Pseudomonas striata*

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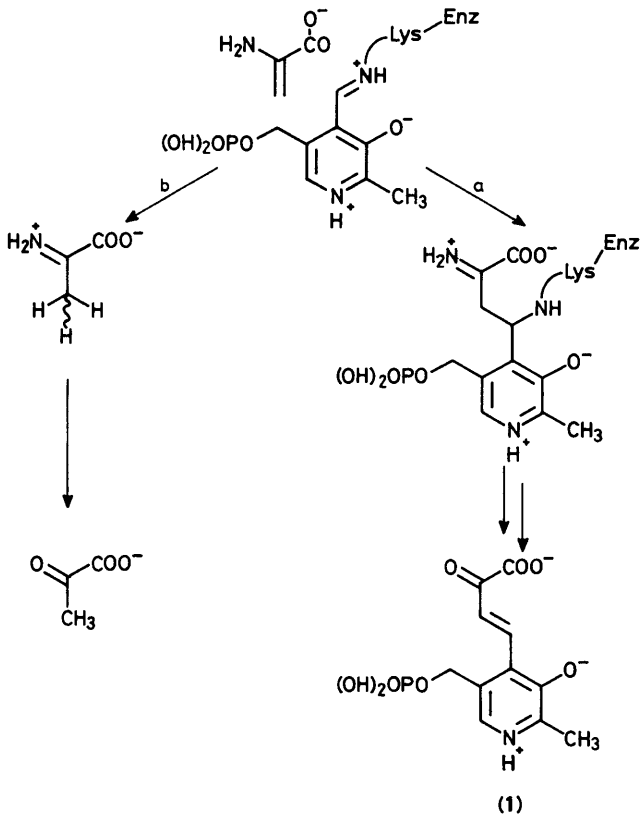
Modest stereoselectivity (15—20% enantiomeric excess) is observed in the protonation of the aminoacrylate product that forms in kinetic competition with suicidal capture of the pyridoxal phosphate (PLP) cofactor by that aminoacrylate in the inactivation of a racemase (*Pseudomonas striata*) with *O*-acetylserines.

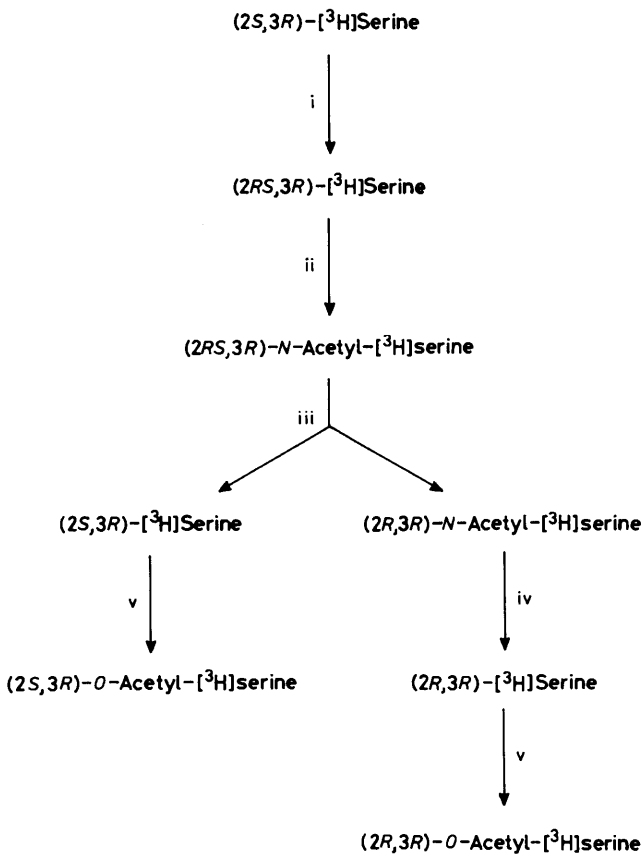
Alanine racemases from *Escherichia coli B* and *Salmonella typhimurium*, and the amino acid racemase from *Pseudomonas striata* are inactivated by D- and L-isomers of β-substituted alanines or serines with almost identical partition ratios, *ca.* 800 catalytic turnovers per inactivation event, in all three cases so far studied.[1—3] We have recently determined that inactivation of the pure racemases from *S. typhimurium* (dad B gene product)[3] and from *P. striata*[2] leads to derivatization of an active site residue not by Michael addition of this residue to the pyridoxal phosphate PLP-bound aminoacrylate but rather by attachment of the C-3 alkylating moiety to both the active site lysine and to the aldehydic carbon of the bound PLP coenzyme. Under denaturing conditions, β-elimination releases the active site lysine and results in formation of 4-[2-methyl-3-hydroxy-5-(phospho-oxymethyl)-4-pyridinyl]-2-oxobut-3-enoic acid, (**1**), first synthesized by Schnackerz *et al.*[4] This adduct must arise from attack of nascent aminoacrylate on the PLP-lysine imine in the active site of the racemases (Scheme 1, pathway a).[5,6] In competition with the coenzyme-inactivating aldol condensation, the aminoacrylate is protonated at C-3 (Scheme 1, pathway b) and the imine then harmlessly hydrolysed to pyruvate and ammonia. In an attempt to determine if these 800 product-forming (aminoacrylate to iminopyruvate) events per inactivation (b/a = 800/1) also occur within the active site, we have used chiral methyl group methodology to analyse the stereo-chemistry at the pyruvate methyl.

Separate incubations of pure *P. striata* racemase[7] were conducted with the four possible chiral 3-tritio-*O*-acetylserine samples (Table 1) in $(^2H)_2O$. The [^{1}H,^{2}H,^{3}H]pyruvate samples obtained were reduced *in situ* to 2*S*-lactate with lactate dehydrogenase (L-LDH) and NADH, which was oxidized to acetic acid and then analysed by the usual enzymatic configurational assay involving activation to acetyl-CoA, then conversion into 2*S*-malate by malate synthase and labilization of the 3*R*-hydrogen by fumarase equilibration[8—10] with [^{14}C]acetate added to give ^{3}H : ^{14}C ratios of 2 to 3.5 for double counting. The required substrates were prepared from (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]serine[11,12] by chemical racemization with pyridoxal and Al^{3+} [13] followed by 2*R*- and 2*S*-isomer separations by *N*-acetylation and acylase treatment[14] (Scheme 2). Finally the samples were *O*-acetylated[15] to yield the four separate suicide substrate isomers.

The results collected in Table 1 show that there is some stereoselectivity in the processing of *O*-acetylserines. The pyruvate is produced by the enzyme with a modest 55 : 45 selectivity in *F* value.[10] These *F* numbers are clearly distinct from the expected 50 : 50 value for racemic processing, and the



Scheme 1



Scheme 2. Typical preparation of 2*R*-isomer of 3*R*-chiral serine. i, Al^{3+}, pyridoxal pH 9.5; ii, Ac_2O–2 M NaOH; iii, acylase resolution; iv, 2 M HCl, 120 °C; v, Ac_2O–AcOH–$HClO_4$.

Table 1. Chirality analysis of lactate produced during *Pseudomonas striata* inactivation with *O*-acetylserines.[a]

Starting isomer of *O*-Acetyl-[3-^{3}H]serine	*F*[b]	E.e.[c]
2*S*,3*R*	54.4	15% *R*
2*R*,3*R*	54.5	16% *R*
2*S*,3*S*	45.0	17% *S*
2*R*,3*S*	44.6	19% *S*

[a] Typically 0.5 mg racemase (12 nmol) and 0.5 mg lactate dehydrogenase (425 units) were dialysed against 10 mM KPO_4–D_2O buffer, pH 7.4, for 20 h with three changes. 0.1 ml of 1 M KPO_4–D_2O, pH 7.4, and 100 μmol NADH were added, then 6 μmol of freshly prepared *O*-acetylserine lyophilized and redissolved in D_2O. The solution (1 ml) was incubated (3.5 h, 37 °C), quenched with acetic acid (0.1 ml), and incubated with Norite (15 min, 50 °C). After centrifugation the supernatant was run through 0.5 ml Dowex 50 H^+ and the lactate further purified by anion exchange chromatography (Dowex 1X10, formate form) using a 0 to 1 M formic acid gradient. [b] The acetic acid sample is mixed with a small amount of [^{14}C]acetic acid and is converted into the coenzyme A ester. Acetyl CoA is then condensed with glyoxylate catalysed by malate synthase, to give malate. The purified malate is incubated with fumarase leading to formation of an equilibrium mixture of malate and fumarate with complete equilibration of the *pro*-3*R*-hydrogen with the solvent. Analysis of ^{3}H : ^{14}C ratio in the lyophilized residue and comparison with that of the starting malate indicate the tritium distribution between the two diastereotopic hydrogen positions of malate; the tritium content of the water is measured also, as a control. We refer to the percent tritium retention in the fumarase action observed in the configuration assay of chiral acetate as *F* value. The numbers given are the average value calculated by two different methods (spillover calculation and simultaneous equations). [c] Enantiomeric excess, $\frac{|F-50|}{29} \times 100$ (%).

results are internally corroborated by complementary pairs of results from the four suicide substrate samples.

We have earlier adduced evidence in favour of a one base mechanism[16] for this racemase, based on partial internal conservation of the alanine α-hydrogen during turnover. To test whether the α-H from the *O*-acetylserine isomers was used by this single active site base in its conjugate acid form to donate a proton back specifically to C-3 of aminoacrylate in the pyruvate-forming sequence, (2*R*,*S*)-*O*-acetyl-[2-^{3}H]serine was allowed to inactive 0.6 mg of racemase (14 nmol) and reduced *in situ* to L-lactate which was then isolated and counted. No significant tritium transfer was detected, ruling out significant internal α-hydrogen return with the *O*-acetylserine substrates. Aminoacrylate protonation could either be slow relative to EnzB–H^+ equilibration with solvent, or different proton donors may be involved in the transfers of H^+ back to C-2 and C-3 of the substrate–PLP adducts. This result leaves in abeyance interpretation of the preferential (55 : 45) aminoacrylate reprotonation geometry until the course of the racemase-mediated elimination has been determined to be *syn* or *anti*. In any event, this study, the first report of experiments probing the stereochemical outcome of the processing of suicide substrates by a racemase, has revealed the stereoselective but not stereospecific reprotonation of equivalent and opposite e.e. from each substrate, consistent with a common intermediate, aminoacrylate, acting as the killer.[17] The 55 : 45 *F* value may reflect almost complete rotation of the aminoacrylate within, or on the way out of, the *P. striata* racemase active site before C-3 protonation.

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