Suicide inhibition of α-oxamine synthases: structures of the covalent adducts of 8-amino-7-oxononanoate synthase with trifluoroalanine

Dmitriy Alexeev,[b] Robert. L. Baxter,*[a] Dominic J. Campopiano,[a] Olivier Kerbarh,[a] Lindsay Sawyer,[b] Nicholas Tomczyk,[a] Rory Watt[a] and Scott P. Webster[a]

The irreversible inhibition of 8-amino-7-oxononanoate synthase by trifluoroalanine involves decarboxylative defluorination of the inhibitor-PLP aldimine followed by attack of the conjugated imine by the amino group of the active site lysine to afford a covalently bound difluorinated intermediate which can subsequently undergo further HF losses and hydrolysis to afford a 2-(pyridoximine phosphate) acetoyl protein adduct.

The α-oxamine synthases are a small but metabolically important family of pyridoxal phosphate (PLP) dependent enzymes which typically catalyse the decarboxylative condensation of amino acids with acyl-CoA derivatives to afford vicinal aminoketones (Scheme 1).[1]
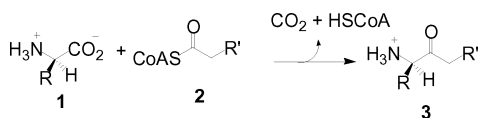
Scheme 1 The α-oxamine synthase reactions; AONS (R = CH$_3$, R′ = (CH$_2$)$_5$CO$_2$H), ALAS (R = H, R′ = (CH$_2$)$_2$CO$_2$H), STP (R = CH$_2$OH, R′ = (CH$_2$)$_7$CH=CH(CH$_2$)$_5$CH$_3$).

The family includes 8-amino-7-oxononanoate synthase (AONS), the first committed enzyme of the biotin biosynthetic pathway;[2] 5-aminolevulinate synthase (ALAS), the enzyme responsible for generating the precursor of the porphyrin rings of hemes in microorganisms and animals;[3] serine palmitoyltransferase (SPT), which catalyses the first step in sphingolipid biosynthesis;[4] and 2-amino-3-ketobutyrate CoA ligase (KBL), which is involved in the threonine degradation pathway.[5] KBL formally catalyses the reverse reaction and is the only member of the family the mechanism of which does not involve a decarboxylation step. Mutations in some of these enzymes have been implicated in a number of human disease states, for example, hereditary sideroblastic anemia caused by a single amino acid substitution in erythroid-specific ALAS (ALAS2)[6] and hereditary sensory neuropathy type I caused by mutations in the SPT subunit-1 (SPTLC1).[7]

The similarity of the reactions involved and the high sequence homology between the different enzymes of the family strongly suggests that all the α-oxamine synthases share a common catalytic mechanism. The crystal structures of bacterial AONS, KBL and

ALAS have now been determined[5,8–10] and it is apparent that all of these have a similar fold and active site geometry. Our understanding of the chemical mechanisms culminates from the results of early radiolabeling studies on *Rhodobacter spheroides* ALAS,[11] and from kinetic studies on *E. coli*[8,9] and *Bacillus sphericus*[12] AONS, and murine ALAS.[13] Crystallographic studies of the product aldimines of *E. coli* AONS and KBL and the substrate aldimine of *Rhodobacter capsulata* ALAS have also been carried out.[5,9,10] In a recent communication we described trapping experiments which provide good evidence for a β-ketoacid intermediate aldimine (AONS-OA) in the AONS reaction sequence.[14] The overall reaction catalysed by AONS can now be conjectured to take place by the route summarised in Scheme 2.

Since biotin synthesis is restricted to plants and microorganisms there has been significant interest in developing specific inhibitors of the enzymes of this pathway as potential biocides.[15] Halogenated substrate analogues have been usefully exploited as inhibitors of PLP dependent enzymes, a therapeutic example being 2,2-difluoromethylornithine which is a suicide inhibitor of ornithine decarboxylase.[16] In the course of our mechanistic studies on AONS we have investigated the suicide inhibition of this enzyme by the substrate analogue, L-trifluoroalanine, and here we describe the characterisation of the covalent enzyme adducts formed, which reveal that the mechanism of inhibition involves a complex sequence of reactions.

It is well established that pyridoxal phosphate (PLP) dependent amino acid racemases, transaminases and decarboxylases can be inhibited by β-halo- and β-polyhalo-analogues of their substrate amino acids. Several inhibitory mechanisms have been described.[17–21] For several enzymes monohaloalanines act as competitive inhibitors, while for others they can lead to enzyme inactivation. The inhibition of alanine racemases by D- and L-fluoroalanines was studied in some depth by Walsh and his coworkers and shown to proceed by two different mechanisms depending on the number of halogen substituents.[17] In all cases C$_\alpha$-H abstraction and halide loss occurs from the corresponding amino acid PLP-imine to afford the unsaturated Schiff's bases **4**–**4b**. In the monofluoro case, **4** undergoes transimination with the active site lysine leading to release of dehydroalanine, which can inactivate the enzyme by attacking the PLP-lysine imine.[17a] Where 3,3-difluoroalanine is the substrate the predicted product, 3-fluorodehydroalanine, is produced but Michael attack of the monofluorinated unsaturated PLP-imine **4a** by the amino group of the active site lysine may lead to reversible formation of the corresponding covalent adduct **5**.[17b] 3,3,3-Trifluoroalanine is a suicide inhibitor and in the case of alanine racemases, the difluorinated unsaturated imine **4b**, formed as above, undergoes

[a]School of Chemistry, Joseph Black Building, The University of Edinburgh, West Mains Road, Edinburgh, UK EH9 3JJ. E-mail: r.baxter@ed.ac.uk; Fax: +44 (0)131 6507155; Tel: +44 (0)131 6504708
[b]School of Biology, Swann Building, The University of Edinburgh, Mayfield Road, Edinburgh, UK EH9 3JR
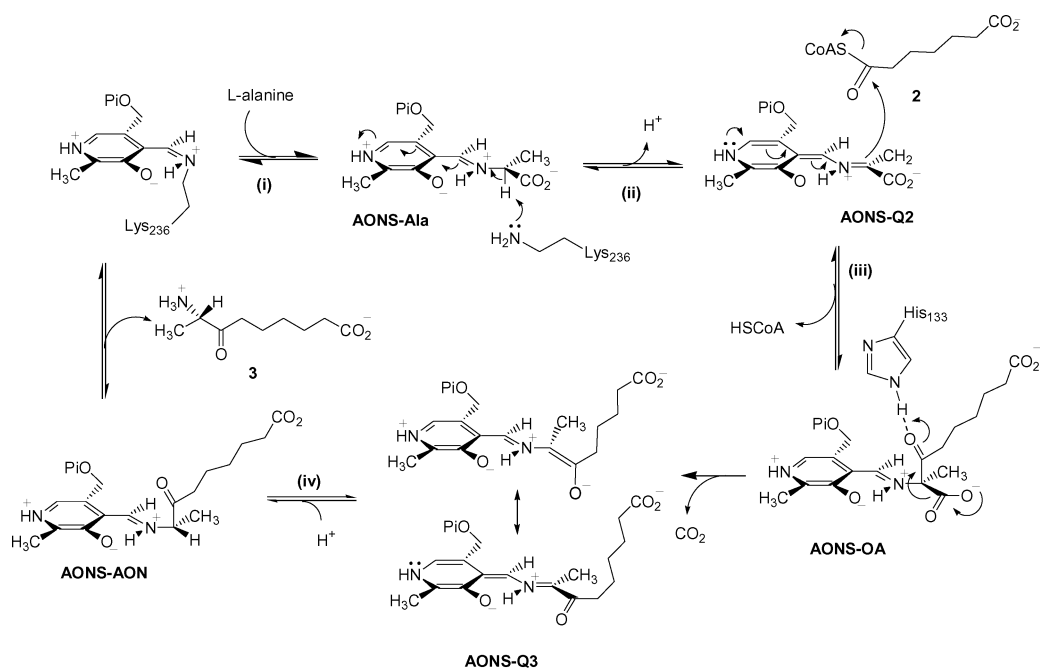
Scheme 2 Catalytic mechanism of the AONS reaction.

Michael attack as before with subsequent fluoride loss to give a stable monofluororinated unsaturated complex **6**. The stability of this structure is surprising and it has been conjectured that complex formation results in the racemase active site becoming impermeable to solvent water.[17b] In contrast the inhibition of cystathione β-lyase by DL-trifluoroalanine results in formation of a complex with loss of all three fluorine atoms suggesting that in this case attack of the complex **6** by water does occur. This leads to further HF loss and formation of the corresponding amide **7**.[18] Validation of this mechanism was provided by elucidation of the crystal structure of the lysine adduct **7** formed when cystathionine β-lyase is incubated with trifluoroalanine.[22]

4 R = R' = H
4a R = H, R' = F
4b R = R' = F

5 R = H, R' = F
5a R = R' = F

6

7
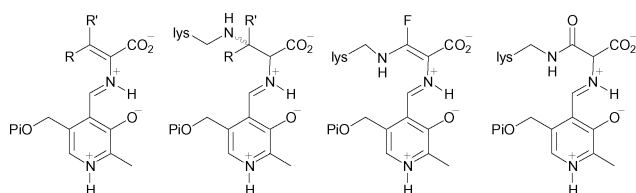
In our studies we found that incubation of *E. coli* AONS with L-trifluoroalanine (50 mM in pH 7.5 phosphate buffer at 25 °C) led to slow inactivation of the enzyme ($t_{\frac{1}{2}}$ ∼20 min) with no detectable activity remaining after 2 h. However the PLP internal aldimine chromophore (λ_{max} 390 & 425 nm) characteristic of the active holoenyme appeared essentially unchanged and no absorption characteristic of formation of an external aldimine was observed. The inhibition could not be reversed by prolonged dialysis with PLP containing buffers. Electrospray mass spectrometry of the inhibited enzyme showed a molecular ion at 41 746 ± 3 amu

corresponding to an increase of 56 ± 3 amu over the native PLP-AONS monomer (41 690 amu). Dialysis of the inactivated protein against 50 mN hydroxylamine removed the PLP cofactor leaving a protein derivative with a monomer molecular weight of 61 ± 3 greater than that of the apo-enzyme showing that the PLP moiety was attached by a labile imine bond. When the initial complex was treated with $NaCNBH_3$ the reduced complex had a molecular mass of 41 751 ± 3 and showed no change on treatment with hydroxylamine confirming the presence of a PLP Schiff's base in the unreduced protein adduct. Although consideration of the AONS mechanism and the previous work on cystathionine β-lyase had led us to predict that an aminomalonoyl adduct of the enzyme might be formed, the mass spectrometry results clearly show that decarboxylation of the substrate skeleton to give an adduct with a mass consistent with structure **9** (predicted mass 41 747 amu) had occurred. This is unexpected since, although a decarboxylation occurs in its catalytic mechanism, AONS itself has no known decarboxylase activity on amino acids. This begs the question at what stage this decarboxylation (presumably with concomitant loss of fluoride) occurs and whether α-H proton abstraction from the trifluoroalanine-PLP imine is the first step in the process. An additional uncertainty was the nature of the enzyme nucleophilic group involved. In alanine racemases and cystathionine β-lyase the nucleophile is the active site lysine which both anchors the PLP and acts as the base for α-H abstraction. However in the analogous adducts formed between 2,2-difluoromethylornithine and ornithine and diaminopimelate decarboxylases, cysteine thiol groups act as the nucleophiles in the Michael addition.[16,23]

To resolve these uncertainties we attempted to prepare the enzyme trifluoroalanine adduct in a crystalline form. However we were unable to crystallise the preformed adduct and so single crystals of holo-AONS were soaked in a solution containing L-trifluoroalanine (10 mM) and PLP (10 mM) in crystallisation

buffer containing 1.6 M ammonium sulfate (1.6 M) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (200 mM) at pH 8.5 and rapidly frozen in liquid nitrogen. The X-ray diffraction data (space group $P3_121$; $a = b = 58.22$ Å, $c = 194.83$ Å, and $\gamma = 120°$ with $\lambda = 1.488$ Å) were collected to a resolution of 1.99 Å. The data were reduced with DENZO/SCALEPACK,[24] and the structure refined with SHELX97 starting from the coordinate set 1bs08.[25] The refinement calculation was interleaved with several rounds of model building with the program O.[26] Water molecules were added using the program SHELXWAT.[25] The final R-factor was 17.9% and the coordinates have been deposited in the Protein Data Bank[27] with accession number 2G6W; the RCSB code is rcsb036763. The electron density map shown in Fig. 1 revealed that the 'active site' lysine (lys236) was modified.
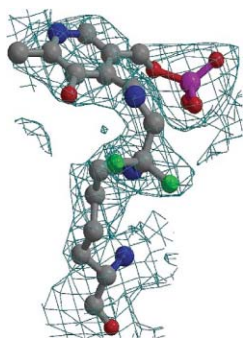

Fig. 1 Electron density around Lys236 calculated with coefficients $3|F_{obs}| - 2|F_{calc}|$, a_{calc} and contoured at 1.6σ. The diagram was prepared with the program O.[26]

A surprising finding was that the crystal structure was not that of the final 2-aminoacetoyl adduct **9**, which contains no fluorine, but rather that of the difluorinated adduct **8**. This implies that decarboxylation occurs at an early stage in the sequence of reactions. A mechanistic rationalisation is shown in Scheme 3. The initial step is formation of the enzyme bound trifluoroalanine-PLP aldimine. Analogy with the mechanisms previously reported for α-haloamino acid inhibition and the normal catalytic mechanism of AONS shown in Scheme 2 would suggest that the first step

after aldimine formation would be proton abstraction by the amino group of lys236 at C-2 which would lead to elimination of a fluoride anion affording **5b**, the double bond of which is activated (by the conjugated carboxylate and imine groups) for Michael attack by the lysine amino group. However, this would require deprotonation of the lysine amino group and that the structure **5b**, which is anchored by the lysine side chain and the PLP group, can then twist to adopt a suitable conformation to facilitate decarboxylation.

The alternative is that decarboxylation and loss of fluoride occurs immediately after aldimine formation (Scheme 3). This gives rise to a reactive 2,3-unsaturated imino species which is attacked by the amino group of lys236. In the crystal phase the reaction terminates at this point. However when the reaction was carried out in solution this complex was not observed. It is reasonable to assume that the next step is HF loss to give a second 2,3-unsaturated imine which is attacked by water to give the intermediate *gem* hydroxyfluoro which then loses HF to give the amide **9**. The lack of stability of the difluorinated intermediate **8** in solution is not unexpected since it has been shown that the AONS structure undergoes significant changes in geometry in the course of its normal catalytic cycle.[9] This inherent flexibility could allow the difluorinated intermediate complex to adopt a suitable conformation for HF elimination and allow attack by a solvent water molecule.

The reason why decarboxylation of trifluoroalanine occurs is unclear since AONS exhibits no native decarboxylase activity with L- or D-alanine. Decarboxylation in the normal mechanism occurs after formation of the ketoacid intermediate (AONS-OA in Scheme 2). In view of the geometric constraints imposed by binding of the pimeloyl derived side chain and the PLP moiety, stabilization of the Dunathan-type intermediate conformation required for a PLP-decarboxylation transition state does not appear likely and we have suggested that the electron sink in decarboxylation may be provided by the protonated ketone.[14]

However, crystallographic studies suggest that, prior to binding of the pimeloyl CoA substrate, there may be sufficient conformational flexibility in the active site to allow the carboxylate of the amino acid-PLP aldimine to interact with a number of potential
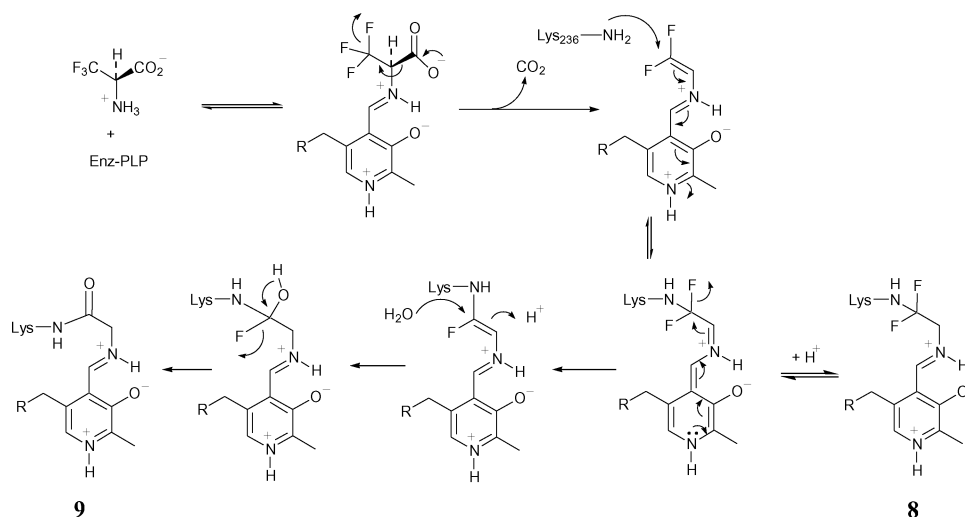
Scheme 3 Formation of AONS adducts with trifluoroalanine ($R = OPO_3^{2-}$).
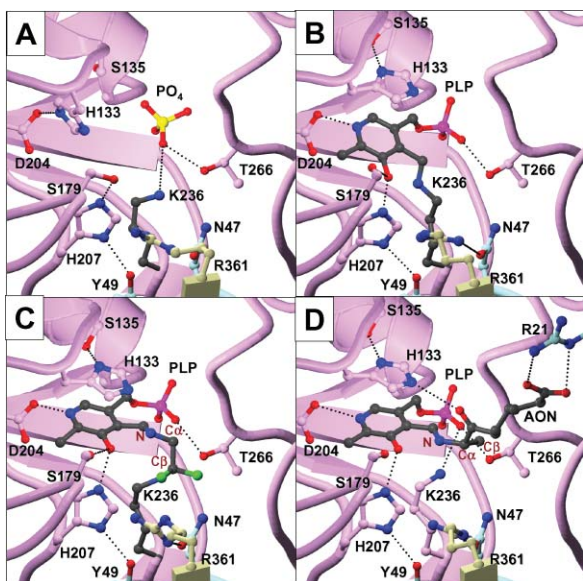
Fig. 2 Comparison of the AONS active site in the apo-enzyme (A), the holo-enzyme (B), the difluoro-adduct (C) and the product aldimine (D). In the difluoro adduct (C), the two fluorine atoms, one of which obscures the NZ of Lys236, are green. The figure was prepared using MOLSCRIPT.[28]

carboxylate docking sites (his133, asn47, arg361). This would allow the $C\alpha$–CO_2 bond to adopt an orientation perpendicular to the plane of the extended PLP ring imine system and thus facilitate decarboxylation. Determination of which of the basic residues interact with the carboxylate in the transition state will require detailed kinetic studies with single residue mutants.

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