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Structural Basis for Phospholyase Activity of a Class III Transaminase Homologue

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Pyridoxal-phosphate (PLP)-dependent enzymes catalyse a remarkable diversity of chemical reactions in nature. A1RDF1 from *Arthrobacter aurescens* TC1 is a fold type I, PLP-dependent enzyme in the class III transaminase (TA) subgroup. Despite sharing 28% sequence identity with its closest structural homologues, including β-alanine:pyruvate and γ-aminobutyrate:α-ketoglutarate TAs, A1RDF1 displayed no TA activity. Activity screening revealed that the enzyme possesses phospholyase (E.C. 4.2.3.2) activity towards *O*-phosphoethanolamine (PEtN), an activity described previously for vertebrate enzymes such as human AGXT2L1, enzymes for which no structure has yet been reported. In order to shed light on the distinctive features of PLP-dependent phospholyases, structures of A1RDF1 in complex with PLP (internal aldimine) and PLP•PEtN (external aldimine) were determined, revealing the basis of substrate binding and the structural factors that distinguish the enzyme from class III homologues that display TA activity.

Pyridoxal-phosphate (PLP)-dependent enzymes catalyse a wide range of chemical reactions, including the racemisation and decarboxylation of amino acids and transamination between amino acid donors and keto acid acceptors,[1,2] and new reactions, including oxidations,[3] continue to be discovered. Some of these enzymes, notably those of the "PLP fold type I" and belonging to the class III transaminase (TA) subgroup, have become extremely useful in biotechnology, because some members possess the ability to form chiral amines from ketone precursors,[4] whereas others catalyse the useful racemisation of amino acid amides.[5,6] In a review in 2015, Steffen-Munsberg and co-workers also drew attention to more obscure and uncharacterised reactions of the class III transaminase group,[1] including roles as phospholyases (E.C. 4.2.3.2). Phospholyases had been identified and partially characterised in early work by Jones[7] and Faulkner[8] in strains of *Erwinia* and *Pseudomonas*.

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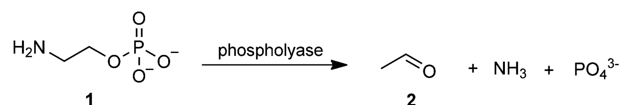

Scheme 1. Transformation of *O*-phosphoethanolamine (PEtN, **1**) by PLP-dependent phospholyases (E.C. 4.2.3.2).

The *Erwinia* enzyme was reported to catalyse the transformation of phosphoethanolamine (PEtN, **1**) to yield acetaldehyde (**2**), ammonia and phosphate (Scheme 1).

The involvement of PLP in the elimination of phosphate from **1** has since been established for vertebrate enzymes such as human AGXT2L1 and AGXT2L2 by the groups of Schaftingen[9] and Peracchi.[10] These enzymes have a role in phospholipid metabolism, and are of interest as playing a role in neuropsychiatric disorders,[9] although no structure of such a PLP-dependent phospholyase has yet been reported. As part of an ongoing study of the structural and catalytic diversity displayed by PLP-dependent enzymes, we cloned the complement of genes encoding predicted transaminase enzymes from the bacterium *Arthrobacter aurescens* TC1[11] into *Escherichia coli*, and many of the genes were expressed in the soluble fraction. From detailed bioinformatics analysis of this enzyme complement by previously described methods,[1] and from direct comparison with the AGXT2L1 sequence,[9,10] it was predicted that the protein with UniProt code A1RDF1 was a phospholyase enzyme. Determination of the structure of such an enzyme would prove valuable because the determinants of phospholyase activity in this PLP enzyme fold type had not previously been described, and might have relevance to studies of the human PLP-dependent phospholyases.

In previous work, an extensive alignment of class III transaminases, containing 12956 sequences, was prepared in order to examine sequence– and structure–function relationships in this family.[1] In that survey, certain combinations of active-site residues (active-site fingerprints) could be related to substrate and reaction specificity of the enzymes of this family. Comparison of the A1RDF1 sequence with these sequences revealed that it was best aligned with the subfamily that contains the α-amino-ε-caprolactam racemases (ACLRs), which catalyse the PLP-dependent racemisation of the named substrate, and which have been applied in the preparative biotransformation of amino acid amides previously.[5,6] None of the active-site fingerprint residues for ACLR activity is present in A1RDF1, however, thus leading to the conclusion that A1RDF1 probably has a different substrate and/or reaction specificity.

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We therefore compared the A1RDF1 sequence with those enzymes with experimentally verified activities and focused especially on active-site residue similarities (Figure S1 in the Supporting Information). This comparison led to the identification of A1RDF1 as a probable phospholyase with PEtN (**1**) as substrate. Two of the highest sequence identities found out of the 201 characterised enzymes in this family were 37.6% with the human PEtN phospholyase (AGXT2L1 UniProt ID: Q8TBG4), and 34.7% with the human 5-phosphohydroxy-L-lysine phospholyase (Q8IUZ5). Although these results were not conclusive, they provided the first suggestion of a phospholyase activity for A1RDF1. This hypothesis was further strengthened by a comparison of the predicted active-site residues with those of AGXT2L1 (Figure S2). A high number of positively charged residues (R90, K412 and R414 in A1RDF1 numbering) suggested that a negatively charged substrate might also be accommodated within the active site of A1RDF1. Additionally, both enzymes share a two-amino-acid deletion in an otherwise highly conserved structural motif. In order to explore the possible activities of A1RDF1 further, it was decided to test the enzyme for ACLR, transaminase and phospholyase activity.

When incubated with L-α-amino-ε-caprolactam and assayed by published procedures,[5,6] A1RDF1 displayed no racemisation activity towards this substrate. When assayed with 25 common and uncommon transaminase amino donors and the two most common amino acceptors, α-ketoglutarate and pyruvate, A1RDF1 displayed no transaminase activity at all. However, when tested with PEtN for β-elimination of phosphate, A1RDF1 showed a specific activity of (71.3 ± 8.6) mU mg^{-1} of protein. Even though this was an order of magnitude lower than the activity found for the human PEtN phospholyase,[10] it can be regarded as a reasonable activity and, from this finding, β-elimination of phosphate may be considered to be at least one of the natural activities of A1RDF1. In order to examine the differences between A1RDF1 and related transaminases more closely, crystal structures of A1RDF1 in complex both with PLP alone (internal aldimine complex) and with PLP•PEtN (external aldimine) were determined to resolutions of 1.50 and 1.87 Å, respectively. Full data collection and refinement statistics can be found in the Supporting Information (Table S1).

In both cases the A1RDF1 structure featured two molecules in the asymmetric unit, forming a dimer with two PLP sites (Figure 1 A). A comparison of the A1RDF1 monomer with known structures carried out with the aid of the DALI server[13] suggested closest structural homology to enzymes of the type I PLP fold class III γ-aminobutyrate:α-ketoglutarate transaminase subgroup such as A1R958, also from *A. aurescens* TC1[11] (28% sequence identity, *Z*-score 46.2, rmsd 2.3 Å), the 2,2-dialkylglycine decarboxylase subgroup, including 1ZC9[14] from *Burkholderia cepacia* (26% sequence identity, *Z*-score 48.3, rmsd 2.0 Å), the *N*-acetylornithine:α-ketoglutarate transaminase group, including 1WKH from *Thermus thermophilus* (28, 47.8, 2.0 Å), and ACLR[15] (3DXV, 31%, 46.9, 2.4 Å). Despite their fairly low sequence homology, superimposition of class III enzymes of transaminase, decarboxylase, ACLR and phospholyase backbone structures did not reveal any significant changes in overall fold, except for a left-handed helix region (residues 69–79, A1R958 numbering), which has a two-amino-acid deletion in A1RDF1. This caused the removal of that structural motif, which is otherwise conserved in the subfamily of transaminase class III enzymes. There was also a loop region, represented by V179–V187 in A1RDF1, which was three amino acids longer in the decarboxylase 1ZC9.

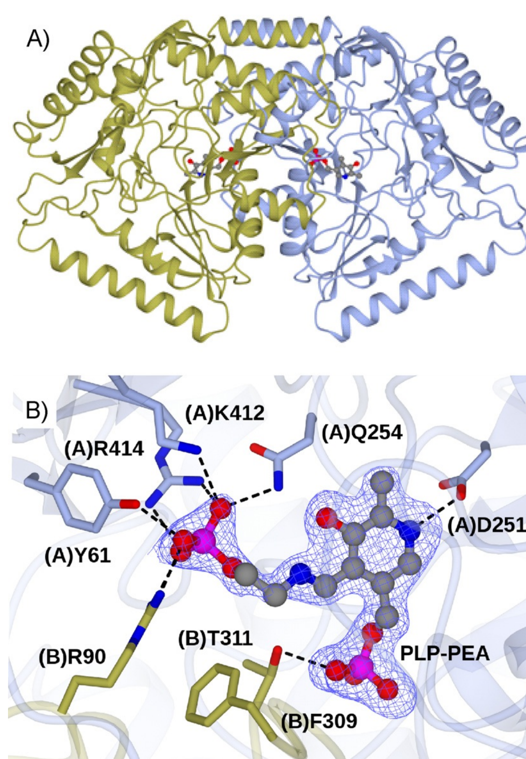

Figure 1. A) Dimer structure of A1RDF1 shown in ribbon format with constituent monomers shown in light blue (monomer A) and gold (monomer B). PLP was observed at the reciprocal dimer interfaces, and is shown in ball-and-stick format with carbon atoms in grey. B) PLP•PEtN external aldimine bound in the active site of phospholyase A1RDF1. Backbone and side chains of monomers A and B are shown in light blue and gold, respectively. PLP•PEtN is shown in ball-and-stick format with the carbon atoms in grey. Electron density corresponds to the F_o-F_c (omit) map obtained before refinement of the ligand and contoured at a level of 3σ. Selected interactions of the ligand with active-site residues are shown by black dashed lines.

In the active site of the PLP complex, the characteristic imine link of the internal aldimine was formed between the side chain of (A)K281 and the electrophilic carbon of PLP. Additionally, a molecule of phosphate, presumably recruited from the growth medium, was observed remote from the PLP molecule, and coordinated by the side chains of a cluster of residues that included Y61, Q254, K412 and R414 from the A subunit, and R90 from the B subunit. The solving of the structure of the PLP•PEtN complex was to reveal that this phosphate was bound at the same site as occupied by the phosphate group of the PEtN ligand, and, indeed, phosphate has been shown to be an inhibitor of the human PEtN phospholyase AGXT2L1.[10] In the PLP•PEtN complex, the bond between the side chain of (A)K281 and PLP was not evident in the electron density; rather, continuous density was observed in the omit map to a ligand that extended into the phosphate binding pocket previously observed in the PLP complex. This was readi-

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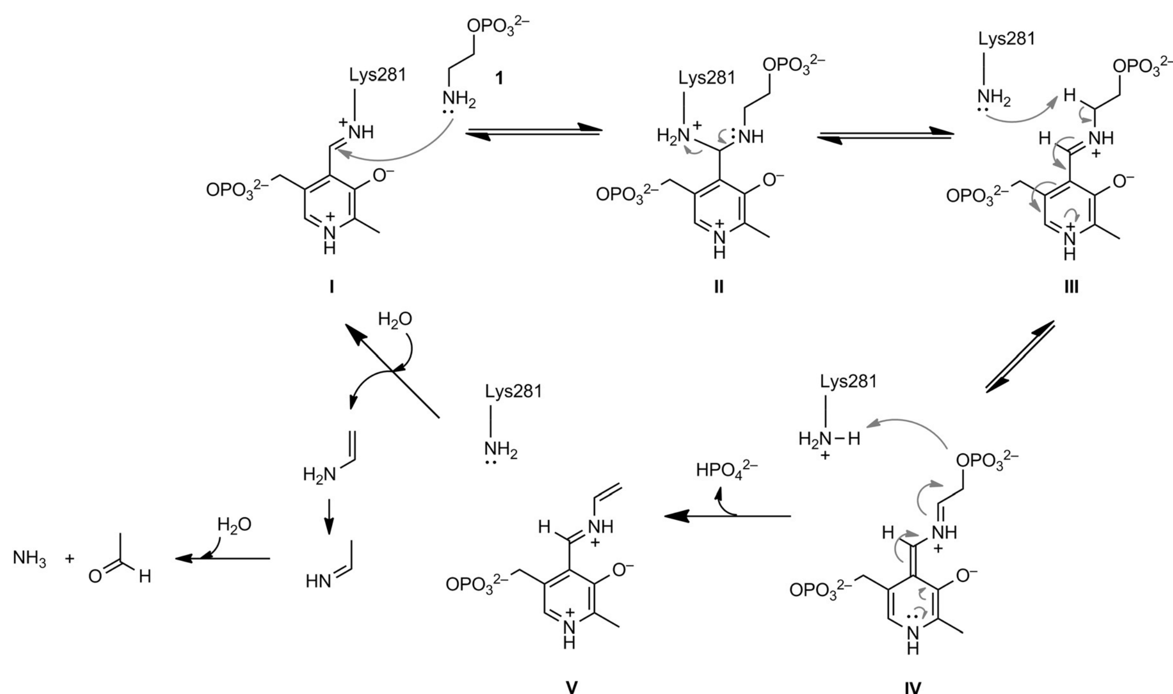

Scheme 2. Mechanism of phospholyase-catalysed elimination of phosphate from PEtN through the action of A1RDF1 (adapted from ref. [8]). **I** = internal aldimine, **II** = *gem*-diamine, **III** = external aldimine, **IV** = quinonoid, **V** = ethyleneamine.

ly modelled as the PLP·PEtN external aldimine (Figure 1B). Each of the free oxygen atoms of the phosphate is tetrahedrally coordinated, making the following interactions with side chains: OAR with the side chain NH2 atom of (A)R90, the NH1 atom of (A)R414 and a water molecule, OAU with the NH1 atom of (B)R90, the phenol of (A)Y61 and a water molecule, and OAT with the side chain NH2 of (A)R414, the NZ atom of (A) K412 and the NE2 amide nitrogen atom of (A)Q254. The phosphate ester oxygen interacts with the NZ atom of the side chain of Lys281, which, in the external aldimine, is now freed from interaction with the PLP molecule.

A mechanism for phospholyase activity in AGXT2L1 was proposed by Schiroli and co-workers,[10] and is adapted in Scheme 2. After formation of the internal aldimine **I** between PLP and K281, PEtN is bound and attacks the imine carbon of **I** to form *gem*-diamine **II**, which releases the catalytic lysine to form the external aldimine **III**. An enzymatic base then deprotonates the terminal carbon of PEtN, resulting in the characteristic quinonoid intermediate **IV**. Although Schiroli, in the absence of a crystal structure of AGXT2L1, was unable to identify the catalytic base, the structure of A1RDF1 suggests that the only protic residue close enough to the terminal carbon of PEtN, at least in this enzyme, is the catalytic lysine K281, at a distance of 3.3 Å.

The side-chain hydroxy group of (B)T311, which also interacts with the PLP phosphate, is at 3.8 Å from the same carbon, but must be considered unlikely to act as a base in view of its role in the binding of PLP. Elimination of phosphate, catalysed through protonation of the phosphoester oxygen by an acidic residue, results in ethyleneamine intermediate **V**, which is hydrolysed to form acetaldehyde, ammonia and PLP. Again, the structure of the complex suggests that the only residue capable of performing the role of protonating the phosphate ester oxygen is K281. This is reminiscent of the role proposed for lysine K69 in the PLP-dependent threonine synthase,[16] which also eliminates phosphate from its substrate (L-homoserine phosphate), although its structural fold (Type II) is very different.

It is clear that, although the overall fold of the class III transaminases has been recruited for phospholyase activity, some residues with established roles remain the same. Each active site possesses an aspartate residue, at position 251 in A1RDF1 and 266 in A1R958, that is thought to protonate the pyridine nitrogen atom during catalysis by transaminases, promoting proton transfer to the exocylic carbon C4′ of PLP.[2] In each enzyme, a glutamine residue (Q254 in A1RDF1 or Q269 in A1R968) interacts with the phenolic oxygen, and a threonine residue (T311/T324) with the PLP phosphate. However, several differences are observed, and these are highlighted by a superimposition of the active sites of the A1RDF1 and A1R958 (4ATQ) external aldimines with PetN and GABA, respectively, shown in Figure 2.

The presence of two arginine residues and one lysine residue in the active site of A1RDF1 suggests its adaptation towards the binding of anionic species such as phosphate; each of these residues is conserved in human AGXT2L1 (Figure S2), and they are thought to provide discrimination over the binding of *O*-sulfoethanolamine, the monoanionic sulfate isostere of **1**, which has a superior leaving group, but is transformed with a k_{cat}/K_M value 1800 times lower than that of PEtN by that

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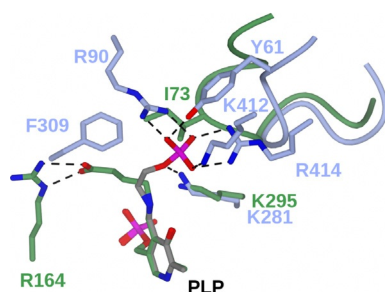

Figure 2. Superimposition of active sites of A1RDF1 and the GABA transaminase A1R958 from *A. aurescens* (4ATQ) in complex with external aldimines formed with PetN and GABA, respectively. Side chains, ligand and annotations for A1RDF1 and A1R958 are in light blue and green, respectively. Selected interactions between ligands and active-site side chains are shown in dashed black lines. The backbone region containing the left-handed helix in A1R958, and which bears residue I73 in that enzyme, are shown in ribbon format.

enzyme.[10] The formation of the dianionic pocket in A1RDF1 occurs at the expense of the short left-handed helix in transaminases such as A1R958. In that enzyme, I73, which protrudes from the short helix, would place a steric constraint on functional group binding, and indeed pushes the GABA chain to the other side of the active site, where the carboxylate is bound by R164 in that enzyme. Reciprocal anion binding sites are therefore formed in the two enzymes. Crucially, the removal of the left-handed helix, and the creation of the dianionic recognition pocket in A1RDF1, also permit the side chain NZ atom of K281 to interact directly with the phosphoester oxygen atom, which would allow it to act as a proton donor to this atom in the elimination of phosphate from the quinonoid intermediate.

The structure of the active site of A1RDF1 confirms the presence of positively charged residues for the recognition of the phosphate moiety of PEtN, which had been predicted from sequence comparison with AGXT2L1.[1,10] The PLP-internal aldimine structure also suggests a possible mode of phosphate inhibition in these phospholyases.[10] In the absence of a structure of the human homologue AGXT2L1, the structure of A1RDF1 presents a useful model for understanding the observed specificity of that enzyme and its role in phospholipid metabolism disorders related to neuropsychiatric disease. It also provides further structural information on the catalytic diversity that has evolved within the class III transaminases, with consequences for studies on enzyme evolution within this family, and also for the engineering of these enzymes for altered activity.

Experimental Section

Details of gene cloning and expression, protein purification, enzyme assay, crystallisation, data collection and building/refinement statistics (including a full data Table S1) can be found in the Supporting Information. Coordinates and structure factor files for the A1RDF1·PLP complex (internal aldimine) and the A1RDF1·PLP·PEtN complex (external aldimine) have been deposited in the Protein DataBank with the IDs 5G4I and 5G4J, respectively.

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Keywords: enzyme catalysis · lyases · phospholyases · pyridoxal phosphate · racemases · transaminases

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