ORGANOFLUORINE METABOLISM IN PLANTS

Mamta Baunthiyal,[a] Abhilasha Pandey[b]

Uttarakhand, India

SUMMARY: This review summarizes the principal studies on biochemical and genetic aspects of the metabolism of organofluorine compounds (organoFs) in plants. Only a few plants are known to biosynthesize the C-F bond and thus form this rare class of fluorinated natural products. Most studies have been done on *Streptomyces cattleya,* which possesses the ability to synthesize organoFs such as fluoroacetate and 4-fluorothreonine from inorganic fluorides. The biosynthetic pathway for the formation of these organoFs in *S. cattleya* and the enzymes involved in their synthesis have been identified and characterized. A gene cluster in *S. cattleya* encodes enzymes involved in the formation of organoFs.

Keywords: Fluorinase; Fluoroacetaldehyde dehydrogenase; Fluoroacetate; 4-Fluorothreonine; Organofluorine compounds; S-adenosylmethionine; *Streptomyces cattleya*.

INTRODUCTION

Organic compounds (organoFs) containing the carbon-fluorine (C-F) bond are extremely rare in living organisms, and in plants only seven distinct examples have been identified.[1] The most widespread of these fluorinated natural products is fluoroacetate (FA), the biosynthesis of which has long been a subject of investigation to determine how C-F bonds are formed biologically. Some plants such as *Acacia,* peanut, castor bean, and pinto bean also have the capability of splitting as well as forming the C-F bond in FA, thereby rendering the plant toxic.[2,3] Moreover, aquatic biota have been shown to convert monoFA into fluorocitrate in water, in which it is further degraded.[4]

Among microbial flora, *Streptomyces cattleya*, a bacterium, has the remarkable ability to biosynthesize the organoFs 4-fluorothreonine (4-FT) and FA from inorganic F.[5] Moreover, bacterial consortia have been isolated and characterized that are capable of degrading fluorobenzoic acids along with 4-fluorophenol and 4-fluorobenzoate.[6]

ENTRY OF F INTO THE CELL

F is taken up by plants from air, water, and soil. The major factors determining the levels of accumulation in plants are plant species, specific plant organ, means of translocation, and growth stage of the plant.[7-8] However, relatively little work has been reported on subcellular localization of F ion into intracellular fluids. In human glioblastoma T98G cells, the nucleus, a perinuclear mitochondria rich cytoplasmic region and the remaining cytoplasm are the three subcellular regions identified for deposition of F administered with boron.[9]

FLUORINATED NATURAL PRODUCTS

Fluorine in the form of inorganic F in minerals is the most abundant halogen in the earth's crust, yet only twelve naturally occurring organoFs have so far been found. [10] Since most inorganic Fs like fluoroapatite and fluorspar in soils have low

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[a]For correspondence: Department of Biotechnology, Govind Ballabh Pant Engineering College, Pauri Garhwal, Uttarakhand, India, 246194. E-mail: mamtabaunthiyal@yahoo.co.in; [b]pandey_abhilasha2003@yahoo.com.

solubility, the F in them has limited bioavailability. Also, the high heat of hydration of F^- greatly limits its ability to participate as a nucleophile in displacement reactions. Moreover, F^- cannot be incorporated into organic compounds via the haloperoxidase reaction since the redox potential required for oxidation of F^- is much greater than that generated by reduction of hydrogen peroxide.

It is also important to note that all organoFs in plants and, to a lesser degree in bacteria, are toxic in general. These include:

Fluoroacetate (FA): FA is the most ubiquitous fluorometabolite was first reported in the South African plant *Dichapetalum cymosum (*gifblaar) by Marais.[11] It is also found in a wide variety of plant species in both arid and tropical regions around the world.[12] Usually occurring in low concentrations in *Nemcia* tea and Guargum[1] and in certain plants such as gifblaar, gastrolobiums, FA accumulates in concentrations as high as 3875 ppm dry weight, and is therefore very poisonous to livestock.[2] Despite high concentrations of FA in many species, not many of the soil and water samples collected from the vicinity of these plants contained FA, thus suggesting its non-persistence in the environment.[2] The toxicity of FA is attributed to its *in vivo* conversion in mitochondria into fluorocitrate[11,13] via condensation of fluoroacetyl CoA with oxaloacetate by the enzyme citrate synthase.[15] Fluorocitrate is a competitive inhibitor of aconitase, a further important enzyme of the Krebs cycle. Experimental support for this mechanism is provided by the X-ray structure of a co-crystal of aconitase with inhibitor bound to enzyme.[14-15]

Fluorinated fatty acids: All fluorinated fatty acids with an even number of C-atoms possess toxicity similar to FA, e.g., fluoropalmitic acid, fluorostearic acid, and fluorooleic acids are the principal toxic compounds of another West African shrub, *D. toxicarium* (ratsbane).[16] ω-Fluorooleic acid is a constituent (3%) of its seed lipids, and the rest appear to be derived from its metabolites. On the other hand, ω-fluorooleic acid originates from the replacement of acetyl-CoA by fluoroacetyl-CoA as the shorter unit in lipid biosynthesis, consistent with fluorine being located only at the terminal (ω) position of lipids. The toxicity of fluorinated fatty acids results from their conversion into FA; nevertheless, some of them are more toxic than FA. The most toxic fluorofatty acid is probably fluorooctanoic acid, but even fluorobutyric acid is approximately four times as toxic as FA.[17]

Nucleocidin: This fluorometabolite was isolated from *Streptomyces calvus.*[18] The first of the two syntheses of nucleocidin confirmed the presence of a F-atom at the 4'position of the ribosyl ring system. There is no structural similarity between FA and nucleocidin, but the structure of the initial fluorinated intermediate in *S. cattleya,* 5'-fluoro-5'deoxy-adenosine, is quite similar to that of nucleocidin; thus the fluorination reaction in *S. calvus* may be similar to reaction in *S. cattleya.* Unfortunately, attempts to re-isolate nucleocidin from cultures of *S. calvus* have been unsuccessful,[19] possibly as a result of the freeze drying methods employed to preserve the culture. Hence, further studies on the biosynthesis of this fluorometabolite may require the organism to be re-isolated from the environment.

2-Fluorobenzoic acid: This organoF is involved in the formation of 2-fluorocatechol through a cyclic peroxide intermediate.[20]

Fluorocitrate: Indirect evidence using extraction with non-polar solvents combined with paper chromatography, inhibition of aconitase, and infra-red spectroscopy, indicated the presence of fluorocitrate along with FA in soybean and crested wheat grass exposed to atmospheric F.[21]

Fluoroactealdehyde: The C-3 and C-4 atoms of 4-fluorothreonine and both C-atoms of FA originate from a common precursor, which has been identified as fluoroacetaldehyde,[21] which is oxidized to FA by a specific NAD^+ dependent transaldolase enzyme.[22]

4-Fluorothreonine (4-FT): The bacterium *S. cattleya* produces FA and 4-FT as secondary metabolites.[4] 4-FT is formed from fluoroacetaldehyde and threonine in a reaction mediated by an unusual pyridoxal phosphate dependent transaldolase enzyme.[23]

Fluoroacetone: Some evidence for the presence of fluoroacetone[24] as a metabolite of the Australian plant *Acacia georginae* has been reported, but there was uncertainty regarding the molecular identity of the volatile organoF metabolite. This work has not yet been re-examined, and the report therefore remains speculative.

ENZYMOLOGY OF ORGANO F METABOLISM

FLUORINASE

S. cattleya can form C-F bonds having evolved as an enzyme able to overcome the chemical challenges of using aqueous F^-. This enzyme is 5'-fluoro-5'deoxyadenosine (5'FDA) synthase (E.C. 2.5.1.63) or fluorinase.[25] It combines S-adenosylmethionine (SAM) and F to form 5'FDA. The various steps of the biosynthetic pathway leading to the formation of FA and 4-FT are now known, and recently several enzymes of this pathway have been identified. [26-30]

To identify the C-F bond-forming enzyme, O'Hagan and colleagues used isotope-labeling studies, but they were unable to reveal the immediate fluorination substrate. Using high-field ^{19}NMR spectroscopy, they conducted a series of experiments incubating cell free extracts with various cofactors, buffers, and F ion, and observed the conversion of ATP and F ion to three organoF molecules. Of these, FA was the most prominent metabolite, next being SAM.[25] At this stage it appeared that SAM was converted into 5'FDA by the fluorination enzyme. Using an HPLC based assay, production of 5'-FDA was monitored directly from SAM, and also fluorinase was purified using standard protocols.

Kinetic analysis of fluorinase revealed a high K_m at 1.9 mM for F ion. This reflects the difficulty of securing F ion from the bulk solution for catalysis on the surface of the enzyme owing to its high heat of hydration. This high K_m also raises questions about the intracellular concentration of F ion within *S. cattleya* cells for the efficient turnover of the enzyme.

Further investigations of the enzyme indicated that the fluorinase is inhibited by SAM analogues such as sinefungin, etc., which are, clearly, competitive inhibitors of the SAM binding sites.

Crystallisation and X-ray structure: The first X-ray differentiation data on the wild type enzyme was obtained and solved to 1.8 Å resolution.[29] The structure revealed a hexamer with the molecular mass of approximately 180–190 kDa, constructed as a dimer of trimers (Figure).

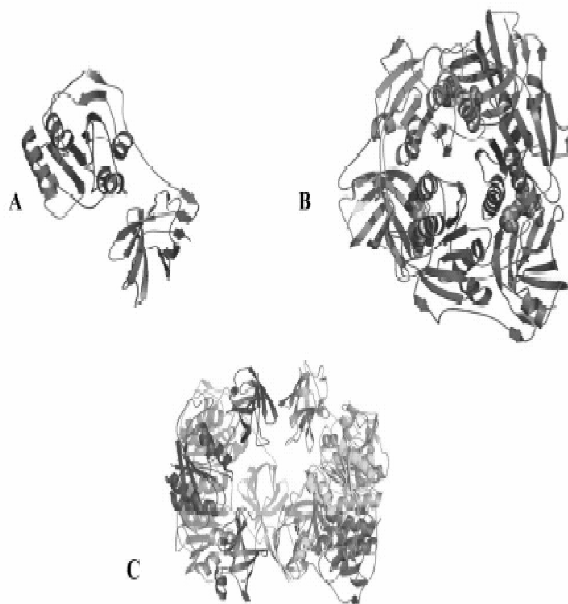

Figure. Structure of florinase (A) monomer (B) trimer with SAM3 bound at subunit surface (C) The overall hexamer structure.[29]

FLUOROACETALDEHYDE DEHYDROGENASE

The first enzyme identified to be directly involved in fluorometabolite biosynthesis, FA dehydrogenase, (E.C. 1.2.1.69),[27] has been found to show the following important characteristics:

Dependence on NAD^+: It is a NAD^+-dependent dehydrogenase.

Mediation of oxidation: It mediates the oxidation of fluoroacetaldehyde to FA.

Stage of dehydrogenase activity: In *S.cattleya,* the production of the enzyme begins in late exponential growth and continues into the stationary phase. These observations are consistent with fluoroacetaldehyde dehydrogenase as an enzyme of secondary metabolism, and its expression coincides with the start of fluorometabolite biosynthesis.[27]

Biochemical Properties: The enzyme is a tetramer of molecular weight approximately 200,000 Da, each monomer unit weighing 55 kDa. The pH optimum of the enzyme was found to be 9.0. Optimum temperature optimum was 45ºC. The reactive site has a thiol group. Addition of EDTA (1mM) increased enzyme activity, indicating that the enzyme may be sensitive to trace amounts of metal ions. The presence of Mg^{2+} (1mM) resulted in an 80% loss of activity, possibly owing to a decreased dissociation rate of NADH from the enzyme.[31]

Amino acid sequence: The N-terminal amino acid sequence of the completely purified fluoroacetaldehyde dehydrogenase was found to be Thr-Val-His-Gln-Ala-Pro-Gly-Ser-Val-Ile-Ser-Leu-Arg-Pro-Pro-Tyr-Asp. This is homologous with the N-terminal sequences (residues 1 to 30) of aldehyde dehydrogenases from *Pseudomonas aeruginosa*[32] and *Deinococcus radiodurans*,[33] 50 and 52%, respectively. Thus, the fluoroacetaldehyde dehydrogenase in *S. cattleya* has similar properties to other aldehyde dehydrogenases, indicating that it is a variant of this class of enzyme.

Substrate specifity of fluoroacetaldehyde dehydrogenase: Of the substrates tested, fluoroacetaldehyde and glycoaldehyde were most efficiently oxidized by the enzyme.[34] Acetaldehyde is a relatively poor substrate, with a K_m that is 10-fold higher than that for fluoroacetaldehyde, indicating that electronic factors are more important for binding than steric properties.

Secondary metabolic enzymes: These are derived by duplication and mutation of enzymes from primary metabolic pathways[34]; hence, it is possible that the fluoroacetaldehyde dehydrogenase enzyme in *S.cattleya* evolved from an aldehyde dehydrogenase that utilized glycoaldehyde, or a similar compound, as the natural substrate.

Genes coding: Since it is common for genes coding for enzymes involved in the biosynthesis of secondary metabolites in bacteria to be clustered,[35] it may now be possible to identify the cluster responsible for FA biosynthesis by targeting the genes coding for this dehydrogenase.

Genes cluster encoding three of the enzymes now identified to be involved in the metabolism of organoFs in *S. cattleya*, has now been identified.[36] These enzymes are F1A catalyzing formation of 5’-fluoro-5’-deoxyadenosine from S-adenosylmethionine and F; F1B catalyzing the conversion of phosphorolytic cleavage of 5’-fluoro-5’-deoxyadenosine to form 5’-fluoro-5’-deoxy-D-ribose-1-phosphate and F1I which is an S-adenosylhomocysteine hydrolase. Besides these, the identified gene cluster also encodes F1k, a thioesterase which confers resistance to FA toxicity in the FA forming strains.

BIOSYNTHESIS OF FLUOROMETABOLITES IN *S. CATTLEYA*

As already noted, the biosynthesis of organoFs by *S. cattleya* NRRL8057 has been examined by high-field NMR spectroscopy.[25,37] Cell suspensions from batch culture harvested at the growth maximum of 4 days were not capable of F uptake or fluorometabolite biosynthesis, but by 6 days had developed an efficient F uptake system and biosynthesized the two fluorometabolites in almost equal proportions. As the harvest age increased, the proportion of FA to 4-FT formed by cell suspensions increased progressively so that at 16 days the cells showed a ratio of 76:26 for the two compounds. Fluorine uptake and fluorometabolite production by cell suspensions were highly dependent on pH, with both processes showing a maximum rate at pH 6.0 but declining rapidly at higher pH. This decrease was considerably marked in the case of F biosynthesis, which was barely detectable at pH 7.5. FA and 4-FT showed only low levels of conversion in cell suspension, thus suggesting that the C- skeleton of neither was derived by metabolism of the other.

This limited interconversion is explicable in terms of a small degree of biological defluorination occurring with each compound, followed by reincorporation of the resulting F ion into the organoF by the active fluorinating system, a phenomenon also noted in the incubation of cell suspensions with a number of other fluorinated biochemical intermediates. By use of ^{19}F NMR spectroscopy, ^{13}C labeled glycine and pyruvates were shown to be incorporated at high levels into FA and 4-FT by resting cells of *S. cattleya* NRRL8057.[37] The labeling patterns illustrate a conversion of glycine via serine into pyruvate, where the C-2 and C-3 of pyruvate contribute both C-atoms of FA and C-3 and C-4 of 4-FT, respectively. These results clearly show that pyruvate is a highly effective precursor of the fluorometabolites resulting in resting cells of *S. cattleya*. It cannot yet be concluded, however, that pyruvate or a closely related metabolite is the substrate of fluorination. Studies using stable and radiolabelled precursors have shown that glycerol is a good precursor in FA biosynthesis.[29,33] The fluorination substrate must therefore be situated between pyruvate and glycerol on the glycolytic pathway.

In a series of experiments using cell free extracts of *S. cattleya*, it was found that the substrates for the formation of 4-FT are fluoroacetaldehyde and L-threonine and that the reaction is strictly dependent on the cofactor pyridoxal phosphate (PLP).[37-38]

FUTURE PROSPECTS

An understanding of the mechanism of enzymatic C-F bond formation could provide new methods for stereospecific incorporation of fluorine into pharmaceutically important compounds such as for breast cancer treatments.[39] Very soon we can hope to have a precise knowledge of deactivation of F by F-tolerant plants and the mechanism of enzymatic C-F bond formation.

The genes coding for fluorinating enzymes may be used to generate recombinant organisms that produce fluorinated derivatives of valuable natural products, for example, antibiotics such as nucleocidin, with altered biological properties. The biotechnological prospects for biofluorination would therefore appear very bright.

CONCLUSION

Since the first reporting of a fluorometabolite in *Dichapetalum cymosum* nearly 68 years ago, progress in understanding the biosynthesis of organoF natural products was limited for some time. The finding by Sanada et al.[5] that the bacterium *S. cattleya* produces 4-FT and FA as secondary metabolites provided a more convenient system to study the biochemistry and enzymology of C-F bond formation. Further research unveiling the mechanism of biosynthesis of organoFs and the enzymology involved is now well underway. The study of F metabolism in plants clearly offers new prospects in bioanalysis of F considering the adverse effects of F pollution as well as the widespread significance of organoFs in commerce in agrochemicals and pharmaceuticals.

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