ENZYMATIC KINETIC PARAMETERS FOR POLYFLUORINATED ALKYL PHOSPHATE HYDROLYSIS BY ALKALINE PHOSPHATASE

Jackson DA[1], Mabury SA[1]

[1] Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Canada.

Introduction

The polyfluoroalkyl phosphate surfactants (PAPs) comprise a large family of commercially used compounds and their recent detection in both the environment and in human blood warrants further investigation into their biotic degradation pathways.

PAPs consist of a hydrophilic phosphate head group bonded to either one (monoPAPs) or two (diPAPs) polyfluorinated groups. PAPs are synthesized by telomerization, which results in an even number of carbon atoms in the fluorinated moiety (usually 4, 6, 8 or 10) and two carbons in an ethylene bridge that links the fluorinated part of the molecule with the phosphate head group. The nomenclature used herein for the monophosphate esters is of the form "x:2 monoPAP" where x equals the number of fluorinated carbons in the structure. A sample structure of the 6:2 monoPAP is given in Figure 1.

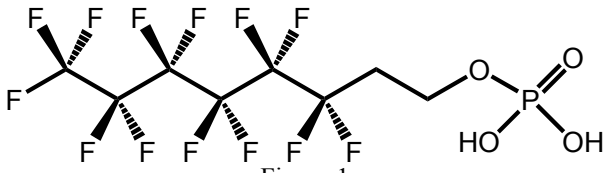

Figure 1

Both monoPAPs and diPAPs are used extensively in the food contact paper industry as their fluorinated chains make them both hydrophobic and lipophobic. Their presence has been detected in the environment[1], and studies have shown their ability to migrate from food packaging into various food simulants[2]. Recently, both monoPAPs and diPAPs were shown to undergo biodegradation in both microbes[3] and rats[4] to produce perfluorinated carboxylic acids (PFCAs) as the end products of metabolism. PFCAs are a well studied class of highly persistent organic pollutants and have been detected ubiquitously in the environment. Due to their extremely long lifetimes and toxicological concerns, detailed studies of the pathways leading to their formation from precursor compounds are needed.

The first step in the biodegradation of x:2 monoPAPs which corresponds to the hydrolysis of the phosphate moiety to produce a fluorotelomer alcohol (x:2 FTOH). Since alkyl phosphate monoesters are extremely slow to hydrolyze under physiological conditions, the process is catalyzed by the alkaline phosphatase enzyme in the small intestine. Alkaline phosphatase is a non specific monoesterase that can hydrolyze a wide variety of both aliphatic and aryl phosphate substrates[5]. Recent studies have demonstrated that esters with better leaving groups are hydrolyzed more efficiently[6]. It was hypothesized that monoPAPs would hydrolyze faster by alkaline phosphatase compared to their fully hydrogenated analogs due to the electron withdrawing fluorinated chain. The exact magnitude of this effect is unclear, however previous studied have shown rate accelerations of at least one order of magnitude by having just one fluorine atom in the beta position[6].

The objective of the present study was to measure the Michaelis Menten enzymatic parameters, k_{cat} and K_M, for the alkaline phosphatase hydrolysis reaction of x:2 monoPAP where x = 4, 6, 8, 10 and compare them for the reactions of their hydrogenated analogs: hexyl, octyl, decyl and dodecyl phosphate, under mildly basic conditions. Although diPAPs were not investigated in the present study, the hydrolysis reaction of diPAPs nonetheless produces one equivalent of monoPAPs in addition to an FTOH. The results from this study will provide data on how efficiently this reaction is likely to occur in biota, as well as provide insight from a physical

organic standpoint on what influence the fluorinated chain has on leaving group ability and how the length of the chain itself alters the rate of the catalytic reaction.

Materials and methods

Synthesis of Hydrogenated and Fluorinated Phosphate Monoesters

Since alkaline phosphatase is strongly inhibited by inorganic phosphate (P_i), it is important that ester starting material is as pure as possible from P_i contamination. Both hydrogenated and polyfluorinated phosphate monoesters were synthesized by the method of Nelson et al[7]. which only produces a monoester product. Using this method, hexyl, octyl and decyl phosphate as well as 4:2, 6:2 and 8:2 monoPAP were synthesized in high purity with acceptable yields. Their structures were confirmed by ^{1}H, ^{19}F and ^{31}P NMR which showed no significant inorganic phosphate contamination. At present, the synthesis of 10:2 monoPAP resulted in too low a yield to be useful for subsequent experiments. Dodecyl phosphoric acid was purchased from Alfa-Aesar. Due to solubility issues, the free acid was converted into its more soluble triethylammonium salt before being used in an experiment.

Determination of Michaelis Constants (K_M) for Phosphate Monoester Hydrolysis by Alkaline Phosphatase

Experiments to determine K_M consisted of a competition kinetics technique utilizing UV-Vis spectroscopy. An aromatic phosphate monoester, p-nitrophenyl phosphate (PNPP), was used as the competitor for all kinetic runs. Its hydrolysis product absorbs light at 405 nm, allowing UV-Vis to determine the initial rate of reaction. Since both the target esters and PNPP compete for the active site of the enzyme, the measured reaction rate decreases as the amount of target ester is increased as indicated by the Michaelis Menten equation for competitive inhibition.

All experiments were carried out using 50 mM Tris buffer, pH 8.5 with a total volume of 1.5 ml. Control Michaelis Menten curves of PNPP hydrolysis in the absence of a target ester were obtained in triplicate using PNPP concentrations ranging from 15 – 500 μM. All PNPP stock solutions were prepared fresh since its background rate of hydrolysis will produce significant quantities of P_i if stored overnight. For competition kinetic analyses, the concentration of target ester was varied, usually between 25 – 1000 μM, while the PNPP concentration was kept constant at 50 μM. Experiments were initiated by the addition of 20 μL bovine intestinal alkaline phosphatase (Sigma-Aldrich P6674-2KU, diluted 1000x).

Data analysis was performed using Sigmaplot 9.0. To obtain the enzymatic parameters V_{max} and K_M for PNPP hydrolysis, a non-linear regression fit to a rectangular hyperbola was performed. To obtain the K_M values for target ester hydrolysis, the complete data set of [PNPP], [target ester] and initial rates including the control data were subjected to a simultaneous non-linear regression and fit to the Michaelis Menten equation for competitive inhibition to obtain the K_M the target ester (either a hydrogenated phosphate ester or x:2 monoPAP).

Since experiments carried out on different days showed variation in PNPP K_M values for an undeterminable reason, the K_M values for the target esters were normalized to the PNPP K_M values as determined concurrently with the competition experiments.

Determination of Turnover Numbers (k_{cat}) for Phosphate Monoester Hydrolysis by Alkaline Phosphatase

Experiments will be performed to determine the k_{cat} values of both the hydrogenated esters and the monoPAPs. The experimental setup will consist of vials containing a concentration of a target ester at far lower concentrations than their K_M values as determined by the previous experiment. After the alkaline phosphatase is added, aliquots will be taken for analysis at various time intervals. Each aliquot will be shaken with ethyl acetate to stop the reaction and extract the alcohol products into the organic phase for GC-MS analysis. By measuring the reaction rates by quantifying product formation, the k_{cat} / K_M ratio can be readily determined from the

simplified Michaelis Menten equation when [S] << K_M. Since the K_M of each target ester has previously been determined, it is a simple matter to solve for k_{cat}.

Results and discussion

The Michaelis constants (K_M) for all but one of the phosphate esters under study have been elucidated and the results are presented graphically in Figure 2. It is important to recall that all values are being presented as the ratio (R) of the experimental K_M of the target ester to the experimental K_M of PNPP as determined by control trials performed concurrently with the competitive kinetic analyses. The raw data obtained from each competition kinetics experiment is given in Table 1.

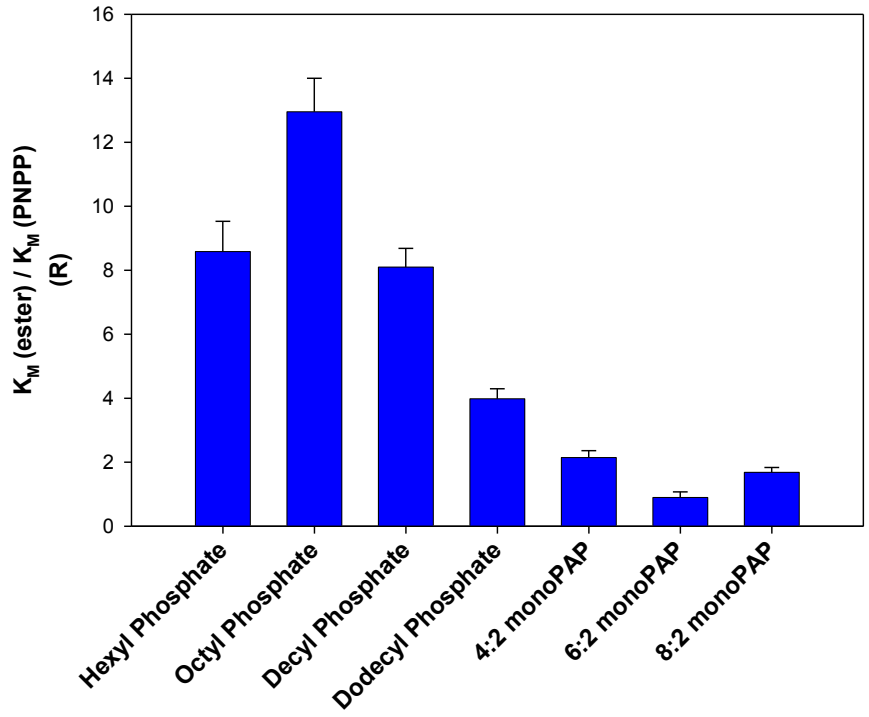

Figure 2

Compound	K_M (experimental, μM)	K_M (PNPP, μM)	R
Hexyl Phosphate	532 ± 53	62 ± 3	8.6 ± 1
Octyl Phosphate	803 ± 52	62 ± 3	13.0 ± 1
Decyl Phosphate	502 ± 27	62 ± 3	8.1 ± 0.6
Dodecyl Phosphate	247 ± 15	62 ± 3	4.0 ± 0.3
4:2 monoPAP	191 ± 17	89 ± 4	2.1 ± 0.2
6:2 monoPAP	80 ± 15	89 ± 4	0.9 ± 0.2
8:2 monoPAP	59 ± 4	35 ± 2	1.7 ± 0.1

Table 1

Two interesting trends are noted from the current results. Firstly, the PAPs all have lower R values than their hydrogenated analogs. This indicates the fluorinated chain increases the catalytic rate compared to a

hydrocarbon chain and they make better substrates for the active site of alkaline phosphatase. This strongly suggests the electron withdrawing ability of the fluorinated chain is stabilizing the departing alkoxide ion despite being separated by the ethylene moiety. However, confirmation of this hypothesis will depend on the results of the upcoming k_{cat} experiments. This is because k_{cat} is included in the K_M term, along with the ability of the substrate to bind to the enzyme reversibly (k_{-1} / k_1). Hence, it is not possible at this time to ascribe all the differences in K_M to a simple effect of pKa differences in the leaving group alcohol, which would become more apparent in the k_{cat} term exclusively.

The second trend noted is a possible chain length effect. For the hydrogenated phosphates, dodecyl phosphate has the lowest R value whereas hexyl and decyl phosphate have statistically the same R value with octyl phosphate having the largest of all. It is unclear why this is the case, although dodecyl phosphate having the longest carbon chain, might have a greater affinity for the enzyme active site through a hydrophobic effect. For the PAPs, 6:2 monoPAP has the lowest R value and an overall trend is hard to discern between them. However, it is interesting to note that the R values of the PAPs occupy a much narrower range of values compared to the hydrogenated phosphates. This might suggest that the leaving group effect outweighs other effects that might be at work, given that changes in chain length should not lead to a significant change in leaving group pKa values.

The upcoming experiments to determine k_{cat} will add substantial value to this work as it will be able to separate out the hydrolysis reaction kinetics from the equilibrium binding constant of the target esters to the active site of the enzyme. The experiments will also give the k_{cat} / K_M ratio, which describes the overall catalytic efficiency of the enzyme, with the most efficient enzymes have ratios approaching 10^9 M^{-1} s^{-1}. Recently, O'Brien et al[6]. found simple alkyl phosphates had k_{cat} / K_M values orders of magnitude lower compared to PNPP for *E. coli* alkaline phosphatase. Since the present study demonstrates that many alkyl phosphates had R values approaching or even less than unity, it is possible much greater differences will be seen in the k_{cat} values for these target esters compared to PNPP and thus the K_M term largely describes the substrate-enzyme binding equilibrium with only a small contribution from the k_{cat} term.

From a toxicological standpoint, this study is significant because it supports the work of D'eon et al[4]. which demonstrated that monoPAPs are capable of being hydrolyzed in the small intestine of a mammal to an FTOH which then degrades further to the corresponding PFCA by metabolic pathways already elucidated. Since high concentrations of phosphatase enzymes are typically found in the small intestine, this is a plausible explanation for the reason why D'eon et al. never detected monoPAP starting material in any bodily tissue after dosing[4].

The results obtained so far in this study would indicate that the PAPs are hydrolyzed faster than their hydrogenated analogs, likely due to a more stable leaving group as imparted by the electron withdrawing fluorinated chain. It is interesting to note this is an example of how the presence of fluorine is able to accelerate a degradation process whereas many environmentally relevant reactions are slowed by fluorination.

Acknowledgements

Funding to DAJ was provided by an Ontario Graduate Scholarship.

References

1. Begley TH, Hsu W, Noonan G, Diachenko G. (2008); *Food Add. Contam.* 25(3): 384-90.
2. D'eon JC, Crozier PW, Furdui VI, Reiner EJ, Libelo EL, Mabury SA. (2009); *Environ. Sci. Technol.* 43(12): 4589-94.
3. Lee H, D'eon JC, Mabury SA. (2010); *Environ. Sci. Technol.* 44(9):3305-10.
4. D'eon JC, Mabury SA. (2010); *Environ. Health Perspect.* 119(3): 344-50.
5. Cleland WW, Hengge AC. (2006); *Chem. Rev.* 106(8): 3252-78.
6. O'Brien PJ, Herschlag D. (2002); *Biochemistry* 41(9): 3207-25.
7. Nelson AK, Toy ADF. (1963); *Inorg. Chem.* 2(4): 775-7.