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^{19}F NMR study on the biological Baeyer–Villiger oxidation of acetophenones†

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The biological Baeyer–Villiger oxidation of acetophenones was studied by ^{19}F nuclear magnetic resonance (NMR). The ^{19}F NMR method was used to characterise the time-dependent conversion of various fluorinated acetophenones in either whole cells of *Pseudomonas fluorescens* ACB or in incubations with purified 4′-hydroxyacetophenone monooxygenase (HAPMO). Whole cells of *P. fluorescens* ACB converted 4′-fluoroacetophenone to 4-fluorophenol and 4′-fluoro-2′-hydroxyacetophenone to 4-fluorocatechol without the accumulation of 4′-fluorophenyl acetates. In contrast to 4-fluorophenol, 4-fluorocatechol was further degraded as evidenced by the formation of stoichiometric amounts of fluoride anion. Purified HAPMO catalysed the strictly NADPH-dependent conversion of fluorinated acetophenones to fluorophenyl acetates. Incubations with HAPMO at pH 6 and 8 showed that the enzymatic Baeyer–Villiger oxidation occurred faster at pH 8 but that the phenyl acetates produced were better stabilised at pH 6. Quantum mechanical characteristics explained why 4′-fluoro-2′-hydroxyphenyl acetate was more sensitive to base-catalysed hydrolysis than 4′-fluorophenyl acetate. All together, ^{19}F NMR proved to be a valid method to evaluate the biological conversion of ring-substituted acetophenones to the corresponding phenyl acetates, which can serve as valuable synthons for further production of industrially relevant chemicals. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 35–42.

Keywords: acetophenone; Baeyer–Villiger oxidation; flavoprotein monooxygenase; ^{19}F NMR; phenyl acetate; *Pseudomonas fluorescens*

Introduction

Ring-substituted phenyl acetates are valuable synthons for the production of fine chemicals and neuroactive pharmaceuticals [11]. These aromatic esters can be produced from treating the corresponding acetophenones with peroxy acids in a Baeyer–Villiger type of reaction [1,4,22]. Because of the laborious clean-up methods required and the danger connected to the use of peroxy acids on a large scale, there is much interest in the development of more sophisticated procedures for the synthesis of substituted phenyl acetates. Production of these compounds by biotechnological processes would provide an attractive environmentally friendly alternative.

Several aerobic microorganisms are capable of utilising acetophenones for their growth [5–7,12–14]. Among these species, *Alcaligenes* sp. strain ACA and *Pseudomonas fluorescens* ACB were active with a wide range of substituted acetophenones [13]. Furthermore, evidence was obtained that in these strains mineralisation of acetophenones involves the initial action of an NADPH-dependent monooxygenase that performs a biological Baeyer–Villiger type of reaction [5,13]. Recently, we succeeded in the purification of this flavoprotein from 4′-hydroxyacetophenone grown cells of *P. fluorescens* ACB and established that the enzyme is active with a wide range of fluorinated acetophenones [17]. This prompted us to address the performance of the purified enzyme and whole-cell preparations for production of phenyl acetates from acetophenones by ^{19}F NMR.

Of all nuclear magnetic resonance (NMR)-observable isotopes, ^{19}F is perhaps the one most convenient for biotransformation studies [20,25]. First, the natural abundance of the ^{19}F isotope is 100% and there are no background signals, because biological systems do not contain NMR-visible fluorinated endogenous compounds. Thus all resonances observed can be ascribed to the fluorinated substrate of interest and its biotransformation products. Second, the ^{19}F nucleus has a broad chemical shift range of about 500 ppm. This is large compared to the chemical shift range of ^{1}H resonances of 15 ppm and of ^{13}C of 250 ppm. Thus, the chemical shift of a ^{19}F nucleus is highly sensitive to its molecular surroundings, resulting in relatively large changes in chemical shifts as a consequence of substrate conversion and reducing the chances of peak overlap. Third, the intrinsic sensitivity of the ^{19}F nucleus is high and is almost comparable to that of ^{1}H. This is an important factor because in whole-cell conversions intermediate products are usually present in relatively low concentrations.

Materials and methods

Chemicals

FAD was purchased from Sigma (St. Louis, MO, USA) and NADPH (grade II) from Merck (Darmstadt, Germany). 4′-Hydroxyacetophenone was from Aldrich (Milwaukee, WI, USA). 2′-Fluoro-, 3′-fluoro-, and 4′-fluoroacetophenone, 2′,4′-difluoroacetophenone, 3′-fluoro- and 4′-fluorophenyl acetate were

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obtained from Fluorochem (Derbyshire, UK) and 4′-fluoro- and 5′-fluoro-2′-hydroxyacetophenone from Lancaster (Lancashire, UK). Fluorinated substrates were more than 95% pure on the basis of ^{19}F NMR analysis. All other chemicals were from Merck and the purest grade available.

Analytical methods

The activity of 4′-hydroxyacetophenone monooxygenase (HAPMO) was determined spectrophotometrically by monitoring the 4′-hydroxyacetophenone-stimulated oxidation of NADPH at 370 nm (ε_{370}=2.7 mM^{-1} cm^{-1}). Activity was measured at 30°C in air-saturated 50 mM potassium phosphate, pH 8.0, containing 250 μM NADPH, and 1 mM 4′-hydroxyacetophenone [7]. The reaction was started by the addition of cell extract or purified enzyme. Specific activities were calculated from initial rate determinations and were corrected for endogenous NADPH oxidase activity. One unit of HAPMO activity is defined as the amount of enzyme that catalyses the oxidation of 1 μmol NADPH per minute under the assay conditions at pH 8.

Conversion of fluorinated acetophenones by whole cells of P. fluorescens ACB

Cells of *P. fluorescens* ACB were grown in mineral medium with 4′-hydroxyacetophenone as sole carbon source at 30°C [13]. Aliquots of 50 ml were centrifuged (10 min, 3000 × *g*, 4°C) and the cells were washed three times with mineral medium [2,3]. To test the biodegradation of 4′-fluoroacetophenone and 4′-fluoro-2′-hydroxyacetophenone, cells were resuspended in 50 ml mineral medium. The optical density at 450 nm of this cell suspension was 1.0. The acetophenone derivate to be tested was added to a final concentration of 1 mM and the cultures were incubated at 30°C on an orbital shaker. To prevent autooxidation of aromatic substrate and products, 1 mM sodium ascorbate (pH 7.0) was added at the start of the incubation. To monitor the time course of the reaction, 2-ml samples were taken at 0, 0.5, 1, 2, 3 and 4 h. After the addition of another 2.5 mM ascorbate, each sample was immediately frozen in liquid nitrogen and stored at −20°C. Before ^{19}F NMR analysis, samples were thawed and centrifuged (5 min, 15,000 × *g*, 4°C) to remove any precipitate formed.

Conversion of fluorinated acetophenones by purified HAPMO from P. fluorescens ACB

HAPMO from *P. fluorescens* ACB was purified by Q-Sepharose and phenyl Sepharose chromatography as described elsewhere [17]. The enzyme was free of esterase activity and more than 90% pure as evidenced by SDS-PAGE [17]. The substrate specificity of HAPMO was studied by ^{19}F NMR product analysis. Tested substrates included 2′-fluoroacetophenone, 3′-fluoroacetophenone, 4′-fluoroacetophenone, 2′,4′-difluoroacetophenone, 4′-fluoro-2′-hydroxyacetophenone and 5′-fluoro-2′-hydroxyacetophenone. Enzymatic incubation mixtures (2 ml) consisted of 0.5 mM fluorinated aromatic substrate, 0.3 mM NADPH, 10 μM FAD, 1 mM ascorbate in air-saturated 50 mM potassium phosphate pH 8.0. Reactions were performed at 30°C and started by the addition of 0.05 U/ml HAPMO unless indicated otherwise. After 1 h of incubation, the reaction samples were frozen in liquid nitrogen and stored at −20°C. Before ^{19}F NMR analysis, samples were thawed and centrifuged (5 min 15,000 × *g*, at 4°C). The pH optimum of the activity of HAPMO with 4′-fluoro-2′-hydroxyacetophenone was determined in 50 mM potassium phosphate pH 5.7 to 9.5. ^{19}F NMR measurements at pH 6.0 and 7.0 were performed with an enzymatic incubation mixture consisting of 1 mM substrate, 1 mM NADPH, 2 mM ascorbate in air-saturated 50 mM potassium phosphate. The reaction was started with 0.38 U/ml HAPMO.

^{19}F NMR product analysis

^{19}F NMR measurements were performed on a Bruker DPX 400 NMR spectrometer, essentially as described elsewhere [23]. The temperature was 7°C. A dedicated 10 mm ^{19}F NMR probehead was used. The spectral width for the ^{19}F NMR measurements was 50,000 Hz. The number of data points used for data acquisition was 32,768. Pulse angles of 30° were used. Between 1000 and 66,000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal-to-noise ratio required. The detection limit of an overnight run (60,000 scans) is 1 μM. The sample volume was 1.6 ml, containing 1.4 ml incubation mixture and 200 μl 0.8 M potassium phosphate pH 7.6 or alternatively, 1.6 ml incubation mixture for pH-dependent studies. For calibration, an insert containing D_2O and a calibrated amount of 4-fluorobenzoate was used, which also served as deuterium lock for locking the magnetic field. Concentrations of the various fluorinated compounds were calculated by comparing the integrals of their ^{19}F NMR resonances to the integral of the 4-fluorobenzoate resonance. Chemical shifts are reported relative to $CFCl_3$. ^{19}F NMR chemical shift values of the various fluorine containing compounds were identified using authentic fluorinated acetophenones and available fluorophenyl acetates. The resonances of fluorophenols and fluorocatechols have been reported previously [19]. ^{1}H decoupling was achieved with a Waltz16 decoupling sequence.

Quantum mechanical calculations

Quantum mechanical molecular orbital calculations were carried out on a Silicon Graphics Indigo using Spartan (version 5.0) (Wavefunction), using the semiempirical AM1 method [8]. The AM1 method has been shown to be suitable and accurate for studies on base-catalysed phenyl acetate hydrolysis [15]. The input geometry was built using the AM1 optimised geometry characteristics reported previously for phenyl acetate and the most stable conformation of the tetrahedral hydroxyl adduct intermediate [15]. For all calculations the geometry was fully optimised. The frontier orbital density for nucleophilic attack was calculated using the equation described by Fukui *et al.* [10]. The relative heat of formation (ΔH_F) for the formation of the tetrahedral intermediate, formed in the rate-limiting step in which a hydroxide anion attacks the carbonyl carbon of the phenyl acetate [15], is calculated as the heat of formation for the tetrahedral intermediate minus the heat of formation of the corresponding phenyl acetate, and presented relative to the lowest ΔH_F value. Atomic charges were calculated on the basis of Mulliken analysis but also from electrostatic potential.

Results

Conversion of fluorinated acetophenones by whole cells of P. fluorescens ACB

Figure 1A and B present the ^{19}F NMR spectra of a 1-h incubation of whole cells of *P. fluorescens* ACB with 4′-fluoroacetophenone

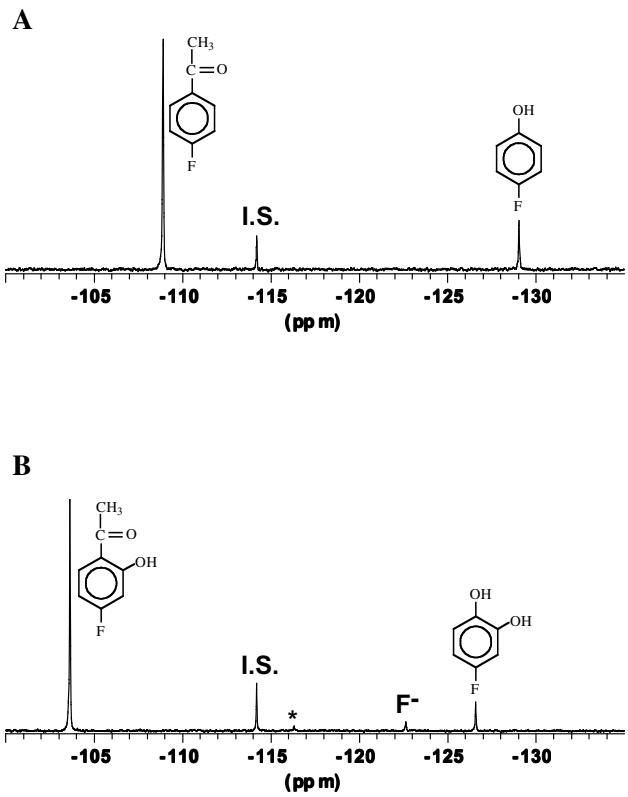

Figure 1 ^{19}F NMR analysis of the conversion of 4′-fluoroacetophenones by whole cells of *P. fluorescens* ACB. (A) ^{19}F NMR spectrum after 1 h incubation with 4′-fluoroacetophenone. (B) ^{19}F NMR spectrum after 1 h incubation with 4′-fluoro-2′-hydroxyacetophenone. The resonance marked I.S. is from the internal standard 4-fluorobenzoate and * indicates a minor impurity in the substrate solution.

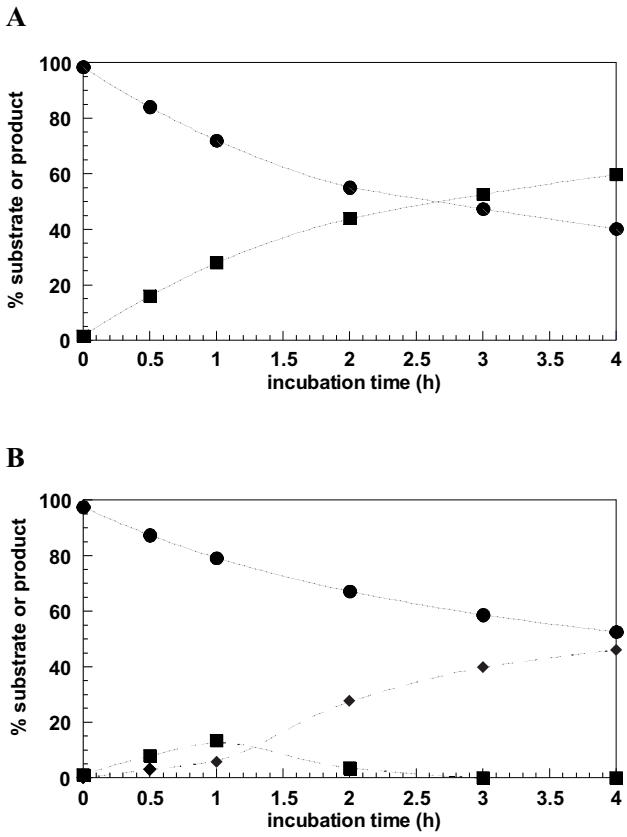

Figure 2 Time-dependent conversion of fluorinated acetophenones by whole cells of *P. fluorescens* ACB. (A) Degradation of 4′-fluoroacetophenone. (●) 4′-fluoroacetophenone; (■) fluorophenol. (B) Degradation of 4′-fluoro-2′-hydroxyacetophenone. (●) 4′-fluoro-2′-hydroxyacetophenone; (■) 4-fluorocatechol; (◆) fluoride anion.

and 4′-fluoro-2′-hydroxyacetophenone, respectively. Both spectra reveal the formation of a single major product with chemical shift at −129.1 ppm (Figure 1A) and at −126.7 ppm (Figure 1B). Based on the chemical shift values of reference compounds (Table 1) these metabolite peaks reflect the formation of 4-fluorophenol from 4′-fluoroacetophenone and of 4-fluorocatechol from 4′-fluoro-2′-hydroxyacetophenone. In the ^{19}F NMR spectrum of the incubation

Table 1 ^{19}F NMR chemical shift values of fluorinated aromatic compounds at pH 7.6

Compound	Product of	^{19}F NMR chemical shift value (ppm)
4-Fluorobenzoate[a]		−114.2
2′-Fluoroacetophenone		−114.5
3′-Fluoroacetophenone		−117.0
4′-Fluoroacetophenone		−108.9
2′,4′-Difluoroacetophenone		−105.5 and −109.1
4′-Fluoro-2′-hydroxyacetophenone		−103.8
5′-Fluoro-2′-hydroxyacetophenone		−128.1
2′-Fluorophenyl acetate[b]	2′-Fluoroacetophenone	−134.0
3′−Fluorophenyl acetate	3′−Fluoroacetophenone	−115.6
4′-Fluorophenyl acetate	4′-Fluoroacetophenone	−120.9
2′,4′-Difluorophenyl acetate[b]	2′,4′-Difluoroacetophenone	−116.5 and −129.0
4′-Fluoro-2′-hydroxyphenyl acetate[b]	4′- or 5′-Fluoro-2′-hydroxyacetophenone	−118.6
2-Fluorophenol[c]	2′-Fluorophenyl acetate	−141.9
3-Fluorophenol[c]	3′-Fluorophenyl acetate	−116.5
4-Fluorophenol[c]	4′-Fluorophenyl acetate	−129.1
2,4-Difluorophenol	2′,4′-Difluorophenyl acetate	−126.1
4-Fluorocatechol[c]	4′- or 5′-Fluoro-2′-hydroxyphenyl acetate	−126.7
Fluoride anion		−122.6

[a]Internal standard.
[b]Not commercially available; estimated on the basis of other products and the known enzymatic reaction.
[c]Peelen *et al.* [19].

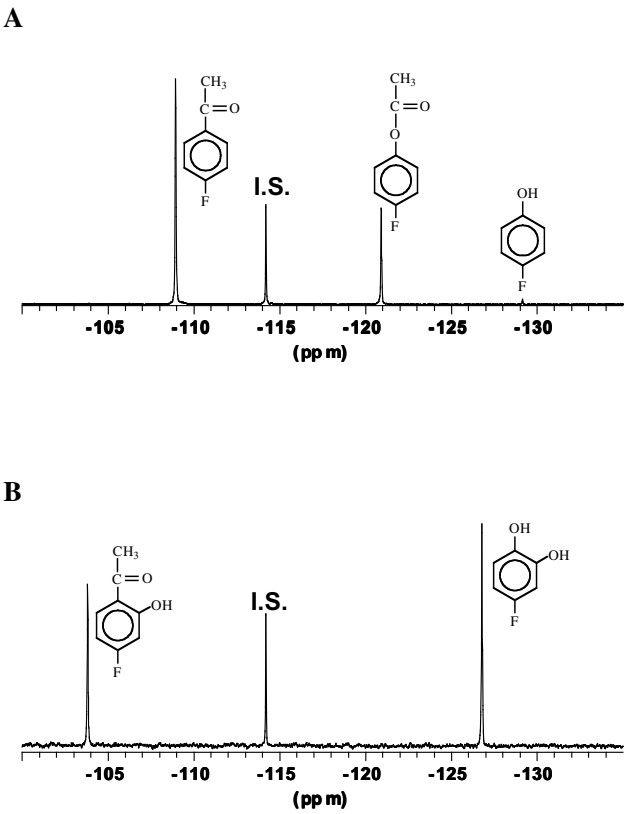

Figure 3 ^{19}F NMR spectral analysis of the conversion of fluorinated acetophenones by 4′-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB. (A) Conversion of 4′-fluoroacetophenone at pH 8.0. (B) Conversion of 4′-fluoro-2′-hydroxyacetophenone at pH 8.0. The resonance marked I.S. is from the internal standard 4-fluorobenzoate.

with 4′-fluoro-2′-hydroxyacetophenone an additional signal at −122.6 ppm, representing fluoride anion formation, is observed (Figure 1B). This fluoride anion formation is not observed in incubations with 4′-fluoroacetophenone (Figure 1A). The time-dependent metabolite patterns of 4′-fluoro- and 4′-fluoro-2′-hydroxyacetophenone, presented in Figure 2A and B corroborate that *P. fluorescens*-mediated conversion of 4′-fluoroacetophenone and 4′-fluoro-2′-hydroxyacetophenone shows a differential behavior with respect to the accumulation of their phenol/catechol-type product and the fluoride anion formation. For 4′-fluoroacetophenone the amount of parent compound converted is fully recovered as 4-fluorophenol (Figure 2A). However, for 4′-fluoro-2′-hydroxyacetophenone formation of 4′-fluorocatechol appears to be a transient phenomenon (Figure 2B). The amount of parent substrate converted is, on the basis of fluoride signal intensity, fully recovered as fluoride anions pointing at further transformation of the 4-fluorocatechol formed. Because frequent ascorbate addition to the samples effectively blocks autooxidation of the fluorocatechol intermediates [2,3], the conversion of 4-fluorocatechol is likely to be mediated by cells of *P. fluorescens* ACB.

The ^{19}F NMR results presented in Figures 1 and 2 indicate that during the time course of the reaction with both acetophenone substrates no other fluorinated products can be detected, pointing to the absence of accumulation of the corresponding 4′-fluorophenyl acetates. This phenomenon can best be ascribed to the swift hydrolysis of these 4′-fluorophenyl acetates by an esterase [13], resulting in formation of 4-fluorophenol and 4-fluorocatechol, the products observed. To eliminate this esterase activity and to provide better possibilities for biosynthesis of the 4′-fluorophenyl acetates from the 4′-fluoroacetophenones, further studies were performed using purified HAPMO.

Conversion of fluorinated acetophenones by purified HAPMO from P. fluorescens ACB

Figure 3A and B present the ^{19}F NMR spectra of a 1-h incubation of 4′-fluoroacetophenone and 4′-fluoro-2′-hydroxyacetophenone with purified HAPMO at pH 8. Comparison of these ^{19}F NMR spectra to those obtained for incubations with whole cells show some marked differences. In contrast to the results obtained with whole cells the incubation of 4′-fluoroacetophenone with purified HAPMO results in formation of a product with its ^{19}F NMR resonance at −120.9 ppm. This product can be identified as 4′-fluorophenyl acetate on the basis of comparison of its ^{19}F NMR chemical shift to that of an authentic standard (Table 1). In these incubations with 4′-fluoroacetophenone and purified HAPMO, formation of 4-fluorophenol was no longer detected (Figure 3A). This confirms that in the absence of esterase activity, HAPMO-catalysed formation of 4′-fluorophenyl acetate from 4′-fluoroacetophenone becomes feasible. In contrast, for 4′-fluoro-2′-hydroxyacetophenone the use of purified HAPMO still results in

Table 2 Rates and yields[a] of the conversion of fluorinated acetophenones by HAPMO

Substrate	pH	k_{cat} (s^{-1})	Phenyl acetate (%)	Phenol/catechol (%)
2′-Fluoroacetophenone	6	n.d.[b]	100.0	0.0
	8	1.1±0.1	89.5	10.5
3′-Fluoroacetophenone	6	n.d.	100.0	0.0
	8	1.1±0.1	90.0	10.0
4′-Fluoroacetophenone	6	n.d.	100.0	0.0
	8	0.7±0.1	96.3	3.7
4′-Fluoro-2′-hydroxyacetophenone	6	n.d.	52.1	47.9
	8	2.9±0.3	0.0	100.0
5′-Fluoro-2′-hydroxyacetophenone	6	n.d.	26.3	73.7
	8	7.3±0.8	0.0	100.0
2′,4′-Difluoroacetophenone	6	n.d.	88.4	11.6
	8	0.5±0.1	64.0	16.0

[a]After 1 h of incubation.
[b]n.d.=not determined.

formation of 4-fluorocatechol as the major product and no accumulation of 4′-fluoro-2′-hydroxyphenyl acetate is observed (Figure 3B). Of interest is that in these incubations of 4′-fluoro-2′-hydroxyacetophenone with purified HAPMO, 4-fluorocatechol formation is no longer accompanied by fluoride anion formation and further degradation of 4-fluorocatechol (Figure 3B). This corroborates that the transformation of 4-fluorocatechol observed in incubations with whole cells is enzyme dependent and not due to chemical degradation/instability of 4-fluorocatechol.

The results obtained for the HAPMO-catalysed conversion of other fluorinated acetophenones were similar to those for 4′-fluoroacetophenone and 4′-fluoro-2′-hydroxyacetophenone (Table 2). Thus, 2′-fluoroacetophenone, 3′-fluoroacetophenone and 2′,4′-difluoroacetophenone were converted to the corresponding phenyl acetate, whereas 5′-fluoro-2′-hydroxyacetophenone was mainly converted to 4-fluorocatechol. The ^{19}F NMR chemical shift values of the fluorinated substrates and their products are presented in Table 1.

pH-dependent HAPMO-catalysed conversion of 4′-fluoro-2′-hydroxyacetophenone

Figure 4 shows the pH-dependent activity of HAPMO with 4′-fluoro-2′-hydroxyacetophenone, indicating the effect of pH on activity of the enzyme. Optimal activity is observed around pH 7, whereas the enzyme is nearly inactive below pH 6 and above pH 9.5. Figure 5A and B present the ^{19}F NMR spectra of incubations of 4′-fluoro-2′-hydroxyacetophenone with purified HAPMO at pH 7.0 and 6.0. These experiments were performed to investigate whether possible base-catalysed hydrolysis of the expected 4′-fluoro-2′-hydroxyphenyl acetate may be responsible for its swift hydrolysis to 4-fluorocatechol at pH 8.0, explaining the absence of its accumulation in these incubations. The results presented in Figure 5A and B indicate that lowering of the pH of the HAPMO incubation with 4′-fluoro-2′-hydroxyacetophenone to pH 6 results in a shift in the nature of the accumulated product, namely a decrease in the amount of 4-fluorocatechol accompanied by an increase in the amount of a fluorinated compound with its resonance at −118.6 ppm, identified as 4′-fluoro-2′-hydroxyphenyl acetate (Table 1). Comparison of the results for 4′-fluoro-2′-hydroxyphenyl acetate to those for 4′-fluorophenyl acetate indicate that the presence of the 2′-hydroxy moiety in 4′-fluoro-2′-hydroxyphenyl acetate makes the compound more sensitive to base-catalysed hydrolysis.

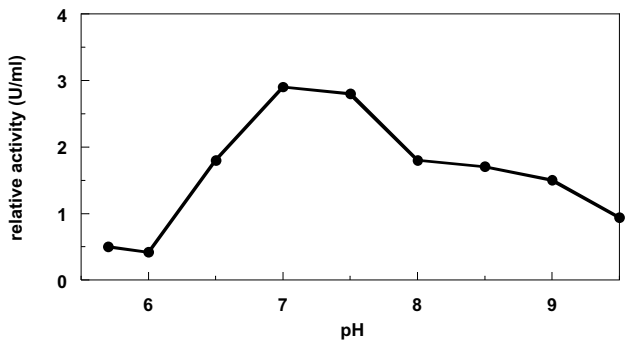

Figure 4 pH-dependent activity of 4′-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB with 4′-fluoro-2′-hydroxyacetophenone.

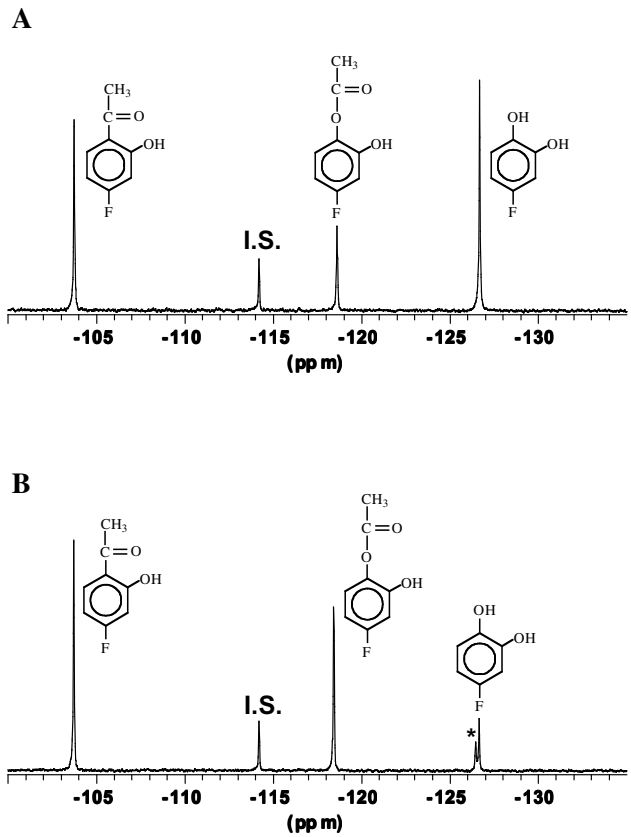

Figure 5 ^{19}F NMR spectral analysis of the pH-dependent conversion of 4′-fluoro-2′-hydroxyacetophenone by 4′-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB at different pH values. (A) pH 7.0, (B) pH 6.0. * Marks an unidentified product.

Quantum mechanical characterisation of the sensitivity of 4′-fluorophenyl acetate and 4′-fluoro-2′-hydroxyphenyl acetate for base-catalysed hydrolysis

To obtain some insight into the factors underlying the differential sensitivity of 4′-fluorophenyl acetate and 4′-

Table 3 AM1-calculated quantum mechanical parameters reflecting possible differences in intrinsic reactivity of 4′-fluorophenyl acetate and 4′-fluoro-2′-hydroxyphenyl acetate for an electrophilic attack by a hydroxyl anion at their carbonyl carbon centre

Parameter	4′-Fluorophenyl acetate	4′-Fluoro-2′-hydroxyphenyl acetate
E(LUMO), eV	−0.08	−0.23
Frontier density for electrophilic attack at C of C=O	0.043	0.024
Mulliken charge at C of C=O	+0.31	+0.30
Atomic charge from electrostatic potential at C of C=O	+0.80	+0.76
Relative ΔH_F for formation of the OH-adduct at C of C=O in kcal/mol	23.9	0[a]

[a]Relative ΔH_F set to zero.

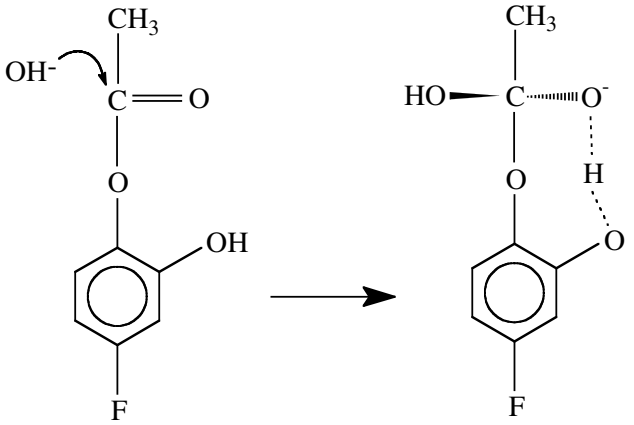

Figure 6 Formation of the proposed tetrahedral intermediate upon base-catalysed hydrolysis of 4′-fluoro-2′-hydroxyphenyl acetate, representing the rate-limiting step of this process [15].

fluoro-2′-hydroxyphenyl acetate for base-catalysed hydrolysis, quantum mechanical calculations were performed. The hydrolysis appeared to be dependent on the pH (Figures 3B and 5A and B) and, thus, on the concentration of the hydroxyl anions. Therefore, the intrinsic reactivity of 4′-fluorophenyl acetate and 4′-fluoro-2′-hydroxyphenyl acetate for a nucleophilic attack by a hydroxyl anion on their carbonyl carbon atom was especially investigated in more detail. This step is known to be the rate-limiting step in base-catalysed hydrolysis of phenyl acetates [15]. Table 3 shows the results obtained. The data indicate that the presence of the hydroxy moiety at C2 in 4′-fluoro-2′-hydroxyphenyl acetate increases the electrophilic reactivity of the carbonyl carbon compared to this carbonyl carbon in 4′-fluorophenyl acetate. This increased reactivity for an attack by a hydroxyl anion is reflected especially by a lower energy of the lowest unoccupied molecular orbital [E(LUMO)]. The relative low-frontier orbital density for attack by a nucleophilic species at the carbon of the carbonyl group observed for both phenyl acetates indicates that the attack of the hydroxyl anion on the carbonyl carbon is not likely to be driven by frontier orbital characteristics. This is in line with the fact that the hydroxyl anion is a hard, not a soft, nucleophile, suggesting its reactivity is dominated by the Coulomb term. However, the calculated charges at the carbonyl carbon were only marginally different between the two 4′-fluorophenyl acetates suggesting that this Coulomb-type reactivity parameter cannot explain the observed differential sensitivity for base-catalysed hydrolysis. The calculated parameter reflecting the differential reactivity of the two 4′-fluorophenyl acetates most clearly is the calculated relative difference in heat of formation for formation of the tetrahedral intermediate hydroxyl adduct of the phenyl acetate (Figure 6), being relatively about 23.9 kcal/mol more favorable for 4′-fluoro-2′-hydroxyphenyl acetate than for 4′-fluorophenyl acetate.

Discussion

Baeyer–Villiger monooxygenases are versatile biocatalysts that have been widely used for the regio- and stereoselective transformation of aliphatic ketones [21,24,26]. Relatively little is known about Baeyer–Villiger monooxygenases that are active with aromatic substrates. Early investigations on bacterial metabolism of acetophenones revealed that in certain *Arthrobacter*, *Nocardia*, *Alcaligenes* and *Pseudomonas* species the ketonic side chain is removed by the successive action of a monooxygenase and an esterase [5–7,12–14]. Our results from ^{19}F NMR analysis on the conversion of fluorinated acetophenones by whole cells of *P. fluorescens* ACB are in agreement with these findings and show that in this strain 4′-fluoroacetophenone is rapidly converted to 4-fluorophenol without the accumulation of 4′-fluorophenyl acetate. Moreover, prolonged incubations with whole cells show that the 4-fluorophenol formed is not further degraded, suggesting that 4′-hydroxyacetophenone grown cells of *P. fluorescens* ACB lack phenol hydroxylase activity. When whole cells of *P. fluorescens* ACB were incubated with 4′-fluoro-2′-hydroxyacetophenone, again no formation of phenyl acetate was observed. However, the 4-fluorocatechol product observed initially was further degraded as evidenced by the formation of stoichiometric amounts of fluoride anion. This suggests that 4′-hydroxyacetophenone-induced cells of *P. fluorescens* ACB contain a dioxygenase that is active with 4-fluorocatechol. Darby *et al.* [7] showed that in 4′-hydroxyacetophenone-induced cells of *Pseudomonas putida* JD1 hydroquinone served as a ring-fission substrate and was cleaved by an oxygen requiring system [7]. Therefore, the question arises whether in *P. fluorescens* ACB, 4-fluorocatechol and hydroquinone are converted by a single enzyme. Hydroquinone dioxygenases acting on haloaromatic substrates have been characterised only recently [16,18,27]. However, in all cases these enzymes appeared to be poorly active with halocatechols.

To obtain more insight into the possibilities for production of fluorinated phenyl acetates from fluorinated acetophenones, studies were performed with purified HAPMO from *P. fluorescens* ACB. ^{19}F NMR product analysis clearly established that conversion of 4′-fluoroacetophenone by purified HAPMO results in accumulation of the expected product 4′-fluorophenyl acetate. This, and the fact that *P. fluorescens* esterase is optimally active around pH 8 [28], confirmed that the HAPMO preparation is free of esterase activity. Enzymatic conversion of 4′-fluoro-2′-hydroxyacetophenone at pH 8.0 did not lead to accumulation of the expected product 4′-fluoro-2′-hydroxyphenyl acetate, but instead resulted in formation of 4-fluorocatechol. Studies of pH dependence indicate that this was due to the base-catalysed non-enzymatic hydrolysis of 4′-fluoro-2′-hydroxyphenyl acetate. Optimal conditions for accumulation of this compound over catechol formation were found at pH 6.0. However, at this pH value, the rate of ester formation was about six times slower than at pH 7, the pH optimum for the HAPMO-catalysed conversion of 4′-fluoro-2′-hydroxyacetophenone.

Quantum mechanical calculations provide some insight into the reason underlying this difference in sensitivity of 4′-fluoro- and 4′-fluoro-2′-hydroxyphenyl acetate for base-catalysed hydrolysis. This analysis was based on a mechanism for base-catalysed phenyl acetate hydrolysis as previously described [15]. This mechanism includes a rate-determining step in which the hydroxyl anion performs a nucleophilic attack on the carbonyl carbon of the phenyl acetate resulting in formation of a tetrahedral reaction pathway intermediate (Figure 6). Especially, calculation of the relative ΔH_F for this reaction step for 4′-fluorophenyl acetate and 4′-fluoro-2′-hydroxyphenylacetate reveals that this rate-limiting reaction step proceeds with a relative heat of formation that is about 23.9 kcal/mol more

favorable for 4′-fluoro-2′-hydroxyphenyl acetate than for 4′-fluorophenyl acetate. It is important that this calculated heat of formation should be considered as a relative rather than an absolute value for the reaction enthalpy. Nevertheless, this higher relative ΔH_F explains the increased sensitivity of 4′-fluoro-2′-hydroxyphenyl acetate for base-catalysed hydrolysis, and, thus, its reduced stability at pH 8 as observed in the present study. Interestingly, the parameters characterising the intrinsic reactivity of the phenyl acetate itself do not reflect such marked differences in reactivity of the parent 4′-fluorophenyl acetates. Thus, the frontier orbital-type characteristics quantifying the relative electrophilicity of the phenyl acetate, the E(LUMO) and the frontier orbital density for electrophilic attack at the carbonyl carbon, vary only slightly. And the Coulomb parameter, i.e., the atomic charge at the carbonyl carbon, known to be important for reactions including hard nucleophiles such as the hydroxyl anion [9], does not vary between 4′-fluorophenyl acetate and 4′-fluoro-2′-hydroxyphenyl acetate. This suggests that the difference in heat of formation, reflecting the higher reactivity of 4′-fluoro-2′-hydroxyphenyl acetate than of 4′-fluorophenyl acetate for base catalysed hydrolysis, may originate from an extra stabilising factor in the tetrahedral intermediate as a result of the 2′-hydroxyl substituent. Clearly additional stabilisation of the tetrahedral reaction intermediate by an intramolecular hydrogen bond between the oxygen of the carbonyl moiety that becomes negatively charged in the tetrahedral reaction intermediate and the proton of the 2′-hydroxy moiety of the benzene ring may explain this stabilising effect of the hydroxyl substituent. The observation that in the optimised geometry of the tetrahedral intermediate the atomic distance between the carbonyl oxygen and the proton of the 2′-hydroxy group amounts to only 0.97 Å supports the formation of this hydrogen bond.

In conclusion, the studies presented here have yielded valuable information on the possibilities for biocatalytic environmentally friendly production of substituted phenyl acetates from substituted acetophenones. Our studies show that the whole-cell approach for this type of biotransformations is only feasible with microbial preparations that lack esterase activity. This might be achieved by inclusion of a suitable esterase inhibitor or in a more sophisticated way by metabolic engineering. Our studies also show that the use of purified enzyme preparations provides a good alternative. However, several bottlenecks remain to be solved to make such an approach economically attractive. These bottlenecks include, among others, the costs of the reduced pyridine nucleotide coenzyme and stabilisation of the aromatic ester of interest.

In this study ^{19}F NMR proved to be a valid method to evaluate the environmentally friendly biological conversion of ring-substituted acetophenones to the corresponding phenyl acetates, which can serve as valuable synthons for further biotechnological production of industrially relevant chemicals.

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