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Investigation of lipase-catalysed hydrolysis of naproxen methyl ester: use of NMR spectroscopy methods to study substrate–enzyme interaction

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Abstract

(±)-2-(6-Methoxy-2-naphthyl)propionic acid methyl ester (methyl ester of Naproxen), the precursor of therapeutically important nonsteroidal anti-inflammatory drugs (NSAIDs) was enantioselectively hydrolysed using as biocatalyst *Candida rugosa* lipase. In research aimed at studing the structure–activity relationship (SAR), NMR spectroscopy methods were employed to identify which Naproxen molecular moiety was essential to the substrate–enzyme interaction. The experimental results, in agreement with previous computer modelling studies and reported kinetic data, gave new information on the enzyme–substrate complex formation in solution.

Keywords: Hydrolysis; Lipase; Naproxen; Structure–activity relationship (SAR); NMR relaxation times

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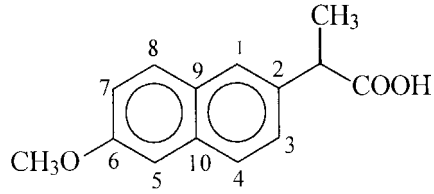
1. Introduction

Hydrolases and in particular microbial lipases have found widespread application as biocatalysts for the solution of synthetic organic problems, in particular in the field of regio- and enantioselective reactions [1,2]. Due to their role in nature as catalysts for the hydrolysis of triglycerides (fats and oils), lipases are ubiquitous in the world of mammals, plants, and micro-organisms such as bacteria and fungi. Although lipases have been designed by nature for the hydrolysis of triglycerides, they are also capable of catalysing the selective hydrolysis and synthesis of unnatural esters with widely differing structures without requiring cofactors. This is also the reason for their widespread application in organic synthesis.

In the last decade the crystallographic data for several enzymes have became available [3–5] providing three-dimensional structures of their active sites. These findings, together with the development of molecular modelling software capable of investigating on the interactions between the lipase amino acid catalytic site and different substrates, offered the possibility of rationalising the lipase–substrate interaction on the basis of the experimental results obtained. On the other hand, in solution ^{1}H-NMR relaxation rate measurements of substrate–lipase complexes yield the evaluation of the motional and environmental features of bonded substrate. Selective irradiation methods can be used throughout the experiments since the selective proton relaxation rate (R_s) is linearly affected by changes in motional correlation time and hence by enzyme-binding interactions, as shown elsewhere [6,7].

Candida rugosa lipase (CRL), one of the most synthetically useful lipase, was employed as catalyst in the enantioselective hydrolysis of (±)-2-(6-methoxy-2-naphthyl)propionic acid methyl esters (methyl ester of Naproxen).



Numbering scheme of Naproxen

This compound, which belongs to the class of nonsteroidal anti-inflammatory drugs (NSAIDs) is used in the treatment of rheumatoid arthritis and osteoarthritis [8]. Only one enantiomer of this class of drugs usually show biocatalytical activity, in particular the *S* enantiomer of Naproxen is 28 times more active than the *R* isomer as an anti-inflammatory agent [9].

Here we present NMR data related to the interaction of (±)-2-(6-methoxy-2-naphthyl)propionic acid with CRL supporting the kinetics hydrolysis experiments.

2. Materials and methods

2.1. Enzyme and substrates

Methyl ester of (±)-2-(6-methoxy-2-naphthyl)propionic acid was obtained from Alfa Wassermann (Karlsrhue, Germany); tributyrin and lipase from *C. rugosa* (Type VII) (commercial-CRL) were purchased from Sigma (St. Louis, MO, USA); another lipase from *C. rugosa* obtained by fermentation in special conditions was the gift of Prof. F. Valero, "Universitat Autonoma de Barcelona" (UAB-CRL).

2.2. Enzymatic reactions

The lipolytic activity was assayed by alkalimetric final titration. The assay mixture, containing 2.5 ml buffer (20 mM phosphate buffer, pH 7.5), 0.5 ml tributyrin, and 0.1 ml enzymatic solution (150 mg/ml), was incubated at 37 °C under magnetic stirring (300 rpm) for 30 min. The reaction mixture was stopped with 2.5 ml acetone/ethanol mixture 1:1 (v/v) and titrated with 0.05 M NaOH in the presence of phenolphthalein as indicator using an automatic burette (Methrom). The protein content was measured with the Sigma kit (Lowry method).

Methyl ester was hydrolysed employing essentially the same procedure reported by Sih et al. [10]. To 100 mg crude CRL in 1 ml of 0.2 M phosphate buffer, pH 7.9, 0.5 mmol ester was added. The resulting suspension was stirred vigorously at 40 °C for 168 h and then centrifuged for 20 min at 600 rpm. The precipitate was washed with 0.2 M phosphate buffer and centrifuged again to collect the water insoluble ester. Supernatant and washing were combined and acidified to pH 2.0 with HCl and the precipitate was collected by filtration to yield the acid.

No hydrolysis was observed at pH 7.5 and 40 °C in the absence of the enzyme.

2.3. Analytical methods

Enzymatic conversion of methyl ester of (±)-2-(6-methoxy-2-naphthyl)propionic acid to the corresponding acid and enantiomeric excesses (e.e. %) were determined by enantioselective normal-phase high-performance liquid chromatography (NP-HPLC). Analytical liquid chromatography was performed on a Waters chromatograph equipped with a Rheody Model 7725i 20 μl injector and two Model M510 solvent-delivery systems. The detector used was a Model M490 programmable multi-wavelength detector (Water Chromatography, Division of Millipore, Milford, MA). Chromatographic data were collected and processed using the Millennium 2010 Chromatography Manager software (Waters Chromatography). The column was a commercially available Chiralcel OD-H chiral stationary phase, 25 cm × 4.6 mm i.d. (Chemical Industries, Tokyo, Japan). The mobile phase was 2% 2-propanol in *n*-hexane (v/v), at a flow rate of 1.0 ml/min. Retention times obtained for the considered compounds were the following: T_1 (*S*)-methyl ester of Naproxen, 4.87 min; T_2 (*R*)-methyl ester of Naproxen, 5.21 min. Chromatographic runs were all monitored by UV detector at 254 nm, at temperature of 25 ± 1 °C.

2.4. NMR studies

Phosphate buffer solutions were prepared in deuterium oxide (Sigma, St. Louis, MO, USA) at pH 7.4, and carefully deoxygenated by three freezing vacuum pumping thawing cycles, immediately followed by sealing off the NMR tube.

All NMR experiments were carried out on a Bruker AM-500 and on a Varian XL 300 spectrometers at the controlled temperature of 300 ± 1 K. Chemical shifts were referenced to external [$^2H_4$]trimethylsilylpropanesulfonate.

Experimental conditions on Bruker AM-500 were: 90° pulse length, 9.0 μs; 12 different τ values from 1 ms to 40 s; repetition rate, 40 s. Experimental condition on Varian XL 300 were: 90° pulse length, 8.5 μs; 12 different τ values from 1 ms to 35 s; repetition rate, 35 s.

Proton spin–lattice relaxation rates were measured with inversion-recovery pulse sequences and calculated by exponential regression analysis of recovery curves of longitudinal magnetisation components. Single and double selective proton spin–lattice relaxation rates were measured with inversion-recovery pulse sequences implemented with DANTE or double DANTE sequences. All relaxation rates were calculated with initial rate approximation [11].

Assignments were performed by two-dimensional COSY experiments and are in line with previous literature data [12].

3. Results and discussion

3.1. Kinetic data

Commercial and UAB lipases were characterised for their lipolytic activity and protein content (Table 1). UAB-CRL showed higher activity and protein content then commercial lipase. This finding was expected given the higher purity of UAB-CRL preparation also in terms of isoform composition [13,14].

Kinetic experiments on (±)-2-(6-methoxy-2-naphthyl)propionate methyl ester in hydrolysis reaction using both lipases as catalysts are reported in Fig. 1.

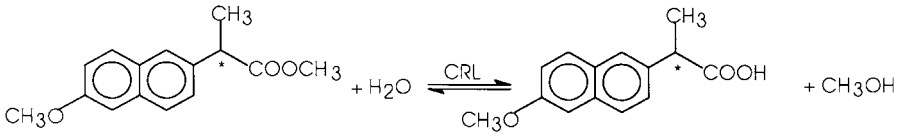


Table 1
Lipases activity

Lipase	Protein content (μg/ml)	Lipolytic activity (μeq acid/min)
Commercial-CRL	18 ± 5	3.7 ± 0.1
UAB-CRL	100 ± 3	11.4 ± 0.1

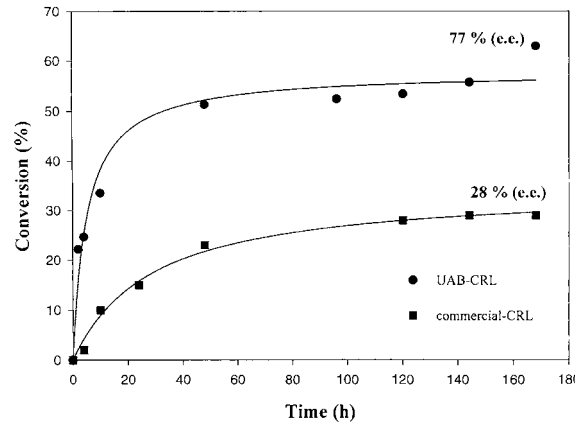


Fig. 1. Hydrolysis reaction of Naproxen methyl ester (0.5 mM) catalysed by two preparation of CRL (100 mg/ml) in buffer solution (pH 7.9). Conversion (%) versus reaction time.

The results showed UAB-CRL was more active than commercial-CRL both in terms of conversion and enantiomeric excess. These data were in agreement with the composition and lipolytic activity of the two different preparations. Moreover, the low hydrolytic rate observed was typical of lipase-catalysed hydrolysis with synthetic substrates and in dispersion systems [15].

3.2. NMR data

The Naproxen proton chemical shifts, relevant assignments and proton relaxation rates are reported in Table 2.

The relaxation values obtained were consistent with a relaxation mechanism mainly determined by ^1H–^1H dipolar interaction as confirmed by R_{nsel}/R_{sel} ratio.

Table 2
^1H NMR chemical shifts, nonselective relaxation rate (R_{nsel}), selective relaxation rate (R_{sel}), relaxation rate fraction ($F = R_{nsel}/R_{sel}$) for 1 mM Naproxen protons in deuteriumoxide–phosphate buffer (0.1 M, pH 7.4, 300 ± 1 K)

δ (ppm)	Assignment	R_{nsel}* (free)	R_{sel}* (free)	F (free)	R_{nsel}* (bound)	R_{sel}* (bound)	F (bound)
7.80	8	0.66	1.48	0.44	0.62	1.63	0.38
7.77	4	0.65	1.43	0.45	0.63	2.72	0.23
7.71	1	0.86	0.49	1.76	0.62	0.76	0.82
7.44	3	0.58	0.44	1.32	0.56	0.98	0.57
7.31	5	0.68	0.67	1.01	0.68	1.14	0.60
7.17	7	0.38	0.65	0.59	0.42	0.66	0.64
3.89	OCH_3	0.74	0.87	0.86	0.76	1.14	0.67
3.74	CH	0.54	nd	nd	0.60	nd	nd
1.44	CH_3	1.58	1.74	0.91	1.56	2.08	0.75

* Errors on the relaxation rates are less than 3%.

The motional correlation times were evaluated by the dipolar interaction energy between protons at fixed distances, σ_{ij} as measured by double selective relaxation rates, according to the equation

$$\sigma_{ij} = R_1^{ij} - R_i^{\mathrm{sel}} = 0.1\frac{\gamma^4\hbar^2}{r_{ij}^6}\left(\frac{6\tau_{ij}}{\omega^2\tau_{ij}^2} - \tau_{ij}\right), \quad (1)$$

where R_1^{ij} is the double selective relaxation rate measured for H_i upon selective excitation of H_i and H_j, R_i^{sel} is the single selective relaxation rate measured for H_i, γ is the proton magnetogyric ratio, r_{ij} is the H_i–H_j internuclear distance, ω is the proton Larmor frequency, $\hbar$ is the reduced Planck's constant and τ_{ij} is the motional correlation time characterising reorientation of the H_i–H_j vector.

In our study this correlation time cannot be identified with the molecular reorientational time because the aromatic moiety is very likely to undergo anisotropic reorientation and the considered internuclear vectors may be rather affected by internal motions.

Infact a certain contribution from a motional anisotropy can be devised since it is known that aromatic molecules as 1,2-dichlorobenzene and 1,2,3-trichlorobenzene exhibit different values for the motional correlation times along the cartesian axes [16].

Consequently, calculations on the basis of Eq. (1), and of the spin lattice relaxation times measurement at different fields [17] provided an effective correlation time τ_c values (Table 3). The τ_c obtained for aromatic protons in absence of lipase showed values in the range 0.55–0.75 ns. In presence of lipase (molar ratio Naproxen/lipase 100:1) the obtained τ_c values were in the range 1.05–4.30 ns, with the higher increase exhibited by H_3, H_5, and H_7 protons. These data clearly indicated that the H_7, H_5, and H_3 protons were the most involved in the enzyme interaction.

On addition of lipase up to lipase:ligand is 0.05 ratio, all chemical shifts were almost unaffected whereas all proton relaxation rates were selectively enhanced, R_{sel} being much more affected, as expected [6], than R_{nsel}.

The selective relaxation rate enhancements could be studied at variable Naproxen concentration while keeping the concentration of lipase constant. In principle such effects might also be determined by the increased viscosity of the medium. However, as already noted elsewhere [18] the fact that $1/\Delta R_{\mathrm{sel}}$ shows linear dependence on the ligand concentration at fixed value of protein concentration demonstrates that the observed phenomena arise from binding to the protein, with consequent slowing

Table 3
Naproxen protons correlation times (τ_c) in absence (a) and in presence (p) of lipase

Proton number	τ_c(a)* (s)	τ_c(p)* (s)
8	6.0×10^{-10}	13.5×10^{-10}
4	6.0×10^{-10}	13.5×10^{-10}
1	5.5×10^{-10}	10.5×10^{-10}
3	7.0×10^{-10}	23.0×10^{-10}
5	7.0×10^{-10}	18.5×10^{-10}
7	7.5×10^{-10}	43.0×10^{-10}

* Error on τ_c values are less than 0.2×10^{-10} (s).

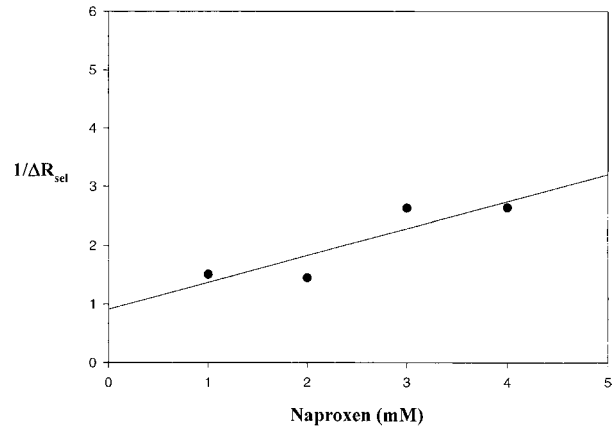


Fig. 2. Behaviour of $1/\Delta R_{sel}$ of Naproxen H_5 proton as function of the substrate concentration in presence of 1 mg/ml lipase in deuterium oxide phosphate buffer (0.1 M, pH 7.4, 300 ± 1 K).

down of molecular motions. Moreover the occurrence of dipole–dipole interactions with the enzyme was expected to contribute to the relaxation rate enhancement. The variation of $1/\Delta R_{sel}$ for H_5 proton is shown in Fig. 2 and allows the evaluation of the apparent association constant (K_{ass}) by extrapolating data to $1/\Delta R_{sel} = 0$, where $[\text{Naproxen}] = -1/K_{ass}$, according to the equation

$$1/\Delta R_i = (1/K_{ass} + [L])(C_M 1/R_{i,b}),$$

where $R_{i,b}$ is the relaxation rate of H_i bound, C_M is the enzyme concentration, and $\Delta R_i = R_i^{obs} - R_i^{free}$.

The calculated value, $K_{ass} = 0.5 \times 10^3\,M^{-1}$, suggests that the Naproxen is not tightly bound by lipase. The proton relaxation scale ($\approx 1\,s^{-1}$) and the observed constant confirm fast chemical exchange of the ligand between the protein bound environment and the bulk such that relaxation rates measured in the presence of lipase are averaged according to the equation

$$R_{obs}^{sel} = p_f R_f^{sel} + p_b R_b^{sel},$$

$$\sigma_{obs}^{ij} = p_f \sigma_f^{ij} + p_b \sigma_b^{ij}$$

the p fractions of ligand in each environment can be approximated by p_b (bond) = protein/ligand and $p_f(\text{free}) = 1 - p_b \approx 1$.

The selective relaxation rate enhancement depends on the increase in the motional correlation time τ_c as well as on the possible increased number of dipolar interaction contributing to the relaxation rate. Such a rate was determined by reduced molecular motions accompanied by dipole–dipole interactions with enzyme protons. It was noticed that the aromatic ring protons were greatly affected, suggesting the mode of binding.

The cross-relaxation rates measured in the presence of the enzyme provided a means of improving the characterisation of the binding interaction, allowing the calculation of the dipolar interaction energies of proton pairs in the bound state.

The cross-relaxation rates change from relatively small positive to large negative values and it is consistent with slowing down of molecular motion from a region where $\omega\tau_c \leqslant 1$ to one where $\omega\tau_c \gg 1$.

4. Conclusions

Since its very first application NMR method has always attracted investigators to the possibility of studying small molecules interacting with macromolecules; unfortunately the NMR approach has always been limited by the exchange rate from the bound state since only fast exchange yields sizeable changes in the NMR parameters. Fast off-rates do not, of course, characterise the usual strong interactions with substrates thus severely limiting NMR applications to systems of most importance. Lipase, on the contrary, does allow NMR investigations of bound substrates, as demonstrated by the present data.

The substrate exchange from the bound state at a rate fast enough to yield measurable selective relaxation rate enhancements thus provides significant dynamic and structural features.

In the case of the investigated substrate the interaction constant measured by NMR relaxation data showed no tight binding between substrate and enzyme. This is in agreement with reported kinetic data in hydrolysis reaction of Naproxen ester. Moreover, the NMR relaxation data showed that only a portion of aromatic ring protons was the most affected by lipase interaction. This kind of interaction between substrate aromatic rings and enzyme was proposed in previous computer modelling studies [19]. The SAR theoretical approach was able to rationalise substrate–enzyme interactions based on enzyme three-dimensional crystal structure and docking studies suggesting some amino acids (particularly Phe345) as playing an important role in determining enzyme–substrate recognition. This finding was confirmed by protein engineering studies [20].

In conclusion this study confirms the π interaction and highlights the substrate moiety directly involved in the enzyme–substrate interaction in solution during the catalytic process.

Acknowledgments

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