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Reversed enantiopreference of *Candida rugosa* lipase supports different modes of binding enantiomers of a chiral acyl donor

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Abstract

Molecular modelling identifies two different productive modes of binding the enantiomers of a 2-methyldecanoic acid ester to the active site of *Candida rugosa* lipase (CRL). The fast reacting *S*-enantiomer occupies the previously identified acyl-binding tunnel of the enzyme, whereas the *R*-enantiomer leaves the tunnel empty. The modelling suggested that if both enantiomers were forced to bind to the active site leaving the tunnel empty, the enzyme would reverse its enantiopreference to become *R*-enantioselective. To test this hypothesis, we designed a structural analogue to 2-methyldecanoic acid, 2-methyl-6-(2-thienyl)hexanoic acid, which was expected to be too bulky to fit its acyl moiety into the acyl-binding tunnel. The CRL-catalysed hydrolysis of the ethyl ester of this substrate resulted in the preferential conversion of the *R*-enantiomer as predicted by molecular modelling. This represents the first kinetic evidence supporting the existence of two different modes of binding the enantiomers of a 2-methyldecanoic acid ester to the active site of CRL. We have shown that a rational 3D based approach in combination with substrate engineering can be used to predict and control the stereochemical outcome of a lipase catalysed reaction.

Keywords: Chiral recognition; Kinetic resolution; *Candida rugosa* lipase; Enantioselective inhibition; 2-Methylalkanoic acid; Enantiopreference; Reversed enantioselectivity

1. Introduction

One of the most commonly used lipases in organic synthesis is *Candida rugosa* lipase (CRL). Apart from being frequently used in kinetic resolution of chiral alcohols, it is also an excellent catalyst for enantioselective transformations of chiral long-chain acyl donors. For instance, an *E*-value of 154 was recently reported in resolution of 2-methyloctanoic acid esterified with eicosanol in cyclohexane [1]. A common strategy in similar reactions is to use an excess of the alcohol to promote product formation and suppress equilibrium conditions. This usually ensures the highest possible *E*-value in the reaction. However, we have found the enantioselectivity of CRL to depend on the concentration of the *n*-alcohol used in esterifications of 2-methylalkanoic acids [2,3] (Scheme 1). At higher alcohol concentrations both reduced enantioselectivities and reaction rates were observed. By reducing the alcohol concentration from 0.9 M to 0.09 M, we were able to raise the *E*-value from 37 to 81 in CRL-catalysed esterification of 2-methyldecanoic acid with *n*-heptanol (Table 1). This phenomenon,

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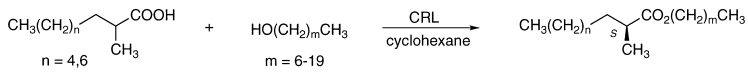


Scheme 1. CRL-catalysed esterifications of 2-methylalkanoic acids.

referred to as enantioselective inhibition, must be controlled and suppressed in order to reach high enantiomeric excess in preparative reactions.

Further kinetic experiments with pure enantiomers of 2-methyldecanoic acid showed that a raised alcohol concentration had a differential effect on V_{max} for the two enantiomers while the K_M-values were equally influenced [2].

To clarify the structural basis for enantioselective inhibition of CRL a molecular modelling study was undertaken [4]. This revealed that the enantiomers of heptyl 2-methyldecanoate, docked into the active site of the lipase as tetrahedral oxyanions, are productively bound to the active site of CRL in two quite different orientations. The fast-reacting *S*-enantiomer may well occupy the acyl-binding tunnel identified in the crystal structures of CRL-inhibitor complexes [5]. This is in line with the proposed rule to predict the enantiopreference of CRL towards 2-substituted carboxylic acids [6]. By contrast, a similar mode of binding the slow-reacting *R*-enantiomer forced His449 of the catalytic triad to adopt a conformation which resulted in the loss of two catalytically essential hydrogen bonds to the transition state. That complex suggested that the *R*-enantiomer is a very poor substrate. This is contradicted by experimental evidence. Instead, the *R*-enantiomer must bind to the active site leaving the tunnel empty ('Hairpin binding mode') allowing the formation of the complete hydrogen bonding network within the active site [4] (Fig. 1). The molecular modelling study also revealed that the fast-reacting *S*-enantiomer formed all catalytically essential hydrogen bonds with both binding modes investigated.

This information allowed us to propose a molecular mechanism explaining how a long-chain alcohol may act as an enantioselective inhibitor of CRL [4]. The alcohol may coordinate to the hydrophobic acyl-binding tunnel of CRL thereby selectively inhibiting the turn-over of the fast reacting *S*-enantiomer, thus resulting in a lower *E*-value in the resolution reaction.

To experimentally test the existence of two binding modes we have now designed a suitable substrate. Our strategy was to tailor a substrate with the long acyl chain ending with a bulky group which was expected to be too big to fit the acyl-binding tunnel, but still structurally analogous to 2-methyldecanoic acid. With such a substrate, the *S*-enantiomer would be forced to bind in the hairpin binding mode similar to that of the *R*-enantiomer in Fig. 1. Molecular modelling suggested that this situation would result in a *R*-enantiopreference of the enzyme [4]. A promising candidate was 2-methyl-6-(2-thienyl)hexanoic acid (Fig. 2).

Here we report the prediction of the enantiopreference of CRL based on molecular modelling. By rational substrate engineering and

Table 1
Apparent enantioselectivity (E) and initial rates in *C. rugosa* lipase catalysed esterification of *rac*-2-methyldecanoic acid (150 mM) with various concentrations of *n*-heptanol [2]

Heptanol (mM)	Initial rate (μmol/min g)	E
900	1.5	37 ± 6
150	6.5	70 ± 11
90	7.7	83 ± 14

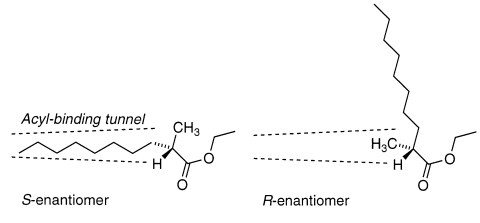


Fig. 1. Schematic view of the active site of CRL illustrating the proposed productive binding modes of ethyl 2-methyldecanoate [4]. The acyl chain of the *S*-enantiomer is bound into the tunnel whereas the *R*-enantiomer is bound into the active site in a hairpin conformation.

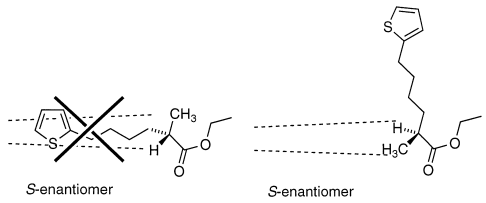


Fig. 2. Imposed binding mode of the designed 2-methylcarboxylic acid ester substrate in CRL. Molecular modelling suggests *R*-preference in this situation [4].

subsequent kinetic analyses we have experimentally challenged this prediction. The results reported herein represent the first kinetic data supporting the existence of two different modes of binding the enantiomers of a chiral acyl donor to the active site of CRL.

2. Materials and methods

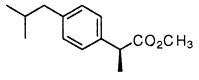
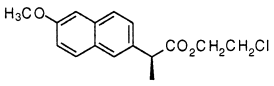
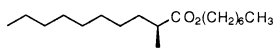
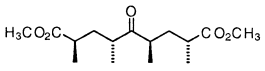
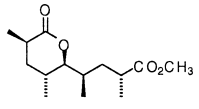
2.1. Enzyme and reagents

Lipase (EC 3.1.1.3) from *C. rugosa* (type VII) was obtained from Sigma (St. Louis, MO, USA). The specific activity was 950 U/mg solid. All chemicals used were of analytical grade. Enantiomerically pure 1-phenylethyl amines were obtained from Fluka (Switzerland). Racemic 2-methyl-6-(2-thienyl)hexanoic acid and 2-methyldecanoic acid were prepared according to a method previously described [7]. Their ethyl esters were prepared by refluxing the corresponding acid during 22 h in absolute ethanol with a few drops of concentrated sulphuric acid present, and were worked up as previously described [8].

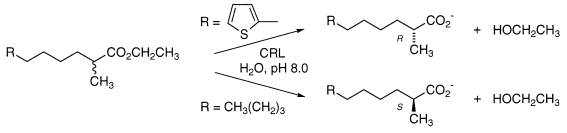
2.2. Hydrolytic reactions

The ethyl ester (0.125 mmol) was added to a water solution (2.5 ml) of gum arabic (5% w/w), $CaCl_2$ (0.2 M) and CRL (26–52 mg/ml, insoluble material was removed by centrifugation), preadjusted to pH 8.0. The mixture was emulsified by sonication for 1 min. The reaction was run under nitrogen in a stirred thermostatted cuvette at 25°C. The pH (8.0) was main-

Table 2
Literature data for the enantiopreference and enantioselectivity of CRL in hydrolysis of 2-methyl substituted carboxylic acid esters [8,14,15]

Substrate[a]	Enantiopreference	*E*	Ref.
	*S*	50	[14]
	*S*	>100	[14]
	*S*	35	[8]
	*R, R*	>100	[15]
	*R, R*	24	[15]

[a] The substrate enantiomer preferred by the enzyme shown.



Scheme 2. Enantiopreference in CRL-catalysed hydrolysis of 2-methylcarboxylic acid esters.

tained automatically with sodium hydroxide (0.1 M), using a Radiometer pH-stat equipped with an ABU 91 autoburette (1 ml) connected to a VIT 90 videotitrator. The reaction was stopped by addition of hydrochloric acid (1.0 M) until pH 1 was reached. The 2-methylcarboxylic acid produced was extracted from the acidified reaction mixture with diethyl ether (6×2 ml) and the combined ether phases were then dried ($MgSO_4$), filtered and evaporated to dryness. The obtained oily liquid was used without further purification for analysis of the enantiomeric excess.

2.3. Enantiomeric excess (ee) and E-values

The ee of the acid was determined by GC after derivatisation to the corresponding diastereomeric phenylethyl amides obtained from reaction with enantiomerically pure 1-phenylethyl amine as previously described [8,9]. The diastereomeric ratio was determined using a Perkin-Elmer 8420 gas chromatograph equipped with a CPSIL-5CB capillary column, 25 m × 0.32 mm I.D., $d_f = 1.2$ μm (Chrompack) using helium as carrier gas (1.7 ml/min, split 1:20). The *E*-values were calculated from the ee of the product (ee_P) and the conversion as described by Chen et al. [10].

2.4. Absolute configuration

The relative configuration was assigned by ^{1}H NMR analysis (500 MHz) of the purified phenylethyl amide derivatives of the acids obtained from the enzymatic hydrolysis reactions in Section 2.2. The absolute configuration of the 2-methyldecanoic acid was assigned based on literature data where both diastereomers of the 2-methyldecanyl MTPA-esters have been identified from their ^{1}H NMR-spectra and then correlated to the optical rotation of the corresponding acid [11]. Optical rotation has previously been used to assign the absolute configuration of the preferred enantiomer of 2-methyldecanoic acid in CRL-catalysed reactions [8]. A range of α-alkylalkanoic acids were prepared by Meyers et al. [12] via asymmetric synthesis from alkylation of chiral 2-oxazolines. The optical rotations reported for all α-alkylalkanoic acids, exclusively, had (*S*)-(+) or (*R*)-(−) configuration.

3. Results and discussion

Methyl substituted carboxylic acids such as Naproxen and Ibuprofen, with a naphtyl group and a substituted phenyl group, respectively, on the stereocentre, are known to react with *S*-enantiopreference (Table 2) [13]. This suggests that both a naphtyl- or a phenyl group do fit into

Table 3
CRL-catalysed hydrolysis of the 2-methylcarboxylic acid ethyl esters, $R(CH_2)_4CH(CH_3)CO_2CH_2CH_3$

R	Initial rate (μmol/min·g)	Conversion (%)	ee_P (%)	*E*	Enantio-preference
	4.1	40.3	46.1	3.6	R
$CH_3(CH_2)_3$	4.6	39.4	13.9	1.4	S

the acyl-binding tunnel of CRL. However, if substituents are present further away from the α-stereocentre in the substrate, CRL has been shown to preferentially catalyse the hydrolysis of the *R*-enantiomer (Table 2). This fits nicely the results reported here for the ethyl ester of 2-methyl-6-(2-thienyl)hexanoic acid.

The ethyl esters of the two racemic 2-methyl-carboxylic acids were prepared and these substrates were individually submitted to CRL-catalysed hydrolysis (Scheme 2).

The reactions were run to 40% conversion and similar specific activities of CRL were obtained with both esters (Table 3). The reactions were stopped and the product ee determined using GC after derivatisation of the acid products to phenylethyl amides using enantiomerically pure 1-phenylethyl amine. The product acids were obtained in 46.1% ee of *R*-configuration and 13.9% ee of *S*-configuration (Table 3). Thus, the introduction of a bulky thienyl group into the substrate resulted in a product with an excess of the opposite enantiomer. The switch in enantiopreference was confirmed for the product acids by assigning the relative configuration of the corresponding phenylethyl amides by ^{1}H NMR (500 MHz). Both ethyl esters gave products with low ee values. This result is not surprising as we have previously shown that the alcohol moiety of a 2-methyl substituted alkanoic acid ester influences the enantioselectivity and that ethyl esters give low *E*-values [16].

To be able to predict, tailor and improve the enantioselectivity of a lipase, it is of fundamental importance to understand the molecular mechanisms involved in chiral discrimination. We have addressed this problem by means of molecular modelling and substrate engineering. The data presented here represent the first kinetic evidence supporting the existence of two different productive modes of binding the enantiomers of a chiral acyl donor to the active site of CRL. This information adds a new dimension to the molecular understanding of chiral recognition by CRL.

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