Remote Interactions Explain the Unusual Regioselectivity of Lipase from *Pseudomonas cepacia* toward the Secondary Hydroxyl of 2'-Deoxynucleosides

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Abstract. Lipase from *Pseudomonas cepacia* (PCL) surprisingly favors acylation of the secondary hydroxyl at the 3'-position over the primary hydroxyl at the 5'-position in 2'-deoxynucleosides by up to >98:1. Molecular modeling found catalytically productive tetrahedral intermediate analogs for both orientations. However, acylation of 3'-hydroxyl places the thymine base in the alternate hydrophobic pocket of PCL's substrate-binding site where it can hydrogen bond to the side chain hydroxyls of Tyr23

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and Tyr29 and the main chain carbonyl of Leu17. Conversely, acylation of the 5'-hydroxyl leaves the thymine base in the solvent where there is no favorable binding to the enzyme. We propose that these remote stabilizing interactions between the thymine base and PCL's substrate-binding site stabilize the 3'-acylation transition state and thus account for the unusual regioselectivity.

Introduction

Many synthetic problems require the chemo- and regioselective modification of hydroxyl groups in complex molecules. This manipulation of diols and polyols is a central matter in synthetic organic chemistry, particularly in the area of carbohydrate and nucleoside chemistry, where preparative procedures typically use repeated protection and deprotection steps.[1] Achieving the required selectivity for different hydroxyl groups is difficult due to their similar reactivity.

Bulky chemical reagents can often functionalize primary hydroxyl groups in the presence of the more hindered secondary hydroxyl groups, since they are typically ten-fold less reactive than primary hydroxyl groups. For example, the trityl or modified trityl groups react selectively at the primary 5'-position of nucleosides over the more hindered secondary 2'- or 3'-positions.[1b] Similarly, bulky acyl groups such as adamantoyl,[2] pivaloyl,[3] mesitoyl,[4] (triphenylmethoxy)acetyl,[5] and several benzoyl derivatives[6] react selectively at the primary 5'-position of nucleosides. In some cases, regioselective acylation of the primary hydroxyls involves more sophisticated reactions, *e.g.,* Mitsunobu conditions or using *N,N*-bis-(2-oxo-oxazolidin-3-yl) phosphorodiammidic chloride.[7]

Hydrolases, and more specifically lipases,[8] can also regioselectively acylate primary hydroxyl groups in the presence of secondary hydroxyl groups in nucleosides,[9] steroids,[10] and carbohydrates.[11] For example, *Candida antarctica* lipase B (CAL-B) catalyzes the selective enzymatic acylation of the primary hydroxyl in several 2'-deoxynucleosides (Scheme 1).[12]

[Insert Scheme1]

Supporting information for this article is available on the WWW under http://chembiochem.org or from the author.

In special cases, chemical methods can favor reactions at a secondary over a primary hydroxyl group. For example, dibutyltin oxide-mediated acylation of diols forms the monoester selectively at the secondary position.[13] Some acylations of carbohydrates favor secondary hydroxyls over primary presumably due to intramolecular hydrogen bonding interactions.[14] Lipase from *Pseudomonas cepacia* (PCL)[†] catalyzes the regioselective acylation of the more hindered secondary 3'-position in β-D-2'-deoxynucleosides (Scheme 1).[12a,12d,12f,16] Identifying the molecular basis of this selectivity using computer-aided molecular modeling is the focus of this paper.

Results

Previous work reported good to excellent 3'-regioselectivity in the acylations of β-D-2'-deoxynucleosides catalyzed by PCL.[12a,12d,12f,16] The best conditions were PCL immobilized on ceramic beads (PCL-C) and using acetonoxime esters as acylating agents in tetrahydrofuran (THF) (Scheme 2). For this study, we focused on the nucleoside with the simplest base (thymine), although other pyrimidine and purine derivatives show similar selectivity. The five acetonoxime esters tested differed in their acyl groups. Three had aliphatic acyl groups (R= Me, Pr, Non) and two contained aromatic rings in the acyl group (R= Ph, CH_2Ph) (Table 1).

[Insert Scheme2]

[Insert Table1]

For all five acyl donors, PCL-C favored acylation of the 3'-hydroxyl of thymidine, but the rate and the degree of regioselectivity varied. Acylations with the short aliphatic acetyl and butanoyl acetonoximes proceeded rapidly but showed moderate regioselectivities (1.3:1 and 6.9:1, respectively; entries 1 and 2,

[†] This enzyme is also known as PSL for *Pseudomonas* species lipase. The microorganism has been recently renamed as *Burkholderia cepacia* (BCL).[15]

Table 1). Acylation with a long aliphatic acyl group - decanoyl - was also rapid, but showed much higher regioselectivity, >98:1 (entry 3, Table 1). Acylations with groups containing an aromatic ring in the acyl chain proceeded approximately ten-fold slower (24–42% conversion after 37–39 h at 30 °C; entries 4 and 6, Table 1), but the regioselectivity was high (>24:1 and >42:1). At 60 °C, the reactions with these aromatic-ring-containing acyl groups were faster and reached similar conversions in half the time (22 h, entries 5 and 7, Table 1), but the regioselectivity decreased significantly (8:1 and 14:1).

The X-ray crystal structure of the open form of PCL (3LIP) shows an active site with three subsites to bind the substrate.[17] Viewing the catalytic triad Asp264-His286-Ser87 from left to right, the three subsites are (Figure 1): a) the large hydrophobic pocket,[18] where the acyl chain binds, which is flanked by residues Val266 and Val267 on the left, Leu167 on the right, Phe119 at the top and Pro113 in the middle; b) the medium sized pocket,[19] where the nucleophile is placed, which is adjacent to the catalytic His286 and Leu287; and c) the alternate hydrophobic pocket[20] to the right of the medium pocket, which can also bind parts of the nucleophile. This alternate hydrophobic pocket lies below the catalytic triad in a narrow region between Ile290, Leu287, Thr18, and Tyr29. Most other lipases, including CAL-B, lack this alternate hydrophobic pocket. During the PCL-catalyzed acylation of thymidine, the nucleoside acts as the nucleophile and thus binds in the medium-sized pocket. Since thymidine is much larger than this pocket, it may extend into other pockets or into the solvent.

[Insert Figure1]

To qualitatively explain the unusual regioselectivity of PCL, we modeled phosphonate analogs of the key intermediates for butanoylation of thymidine. Phosphonates both mimic key features of the intermediates in hydrolysis reactions and allow using the computationally simpler molecular mechanics approach. This approach focuses on how the substrate fits in the enzyme, but may omit subtle transition state details.

We started with a simplified phosphonate that mimicked the tetrahedral intermediate for butanoylation of ethanol (Scheme 3). Geometry optimization yielded a structure containing all five catalytically essential hydrogen bonds. To model the nucleoside substrates, we replaced the ethoxy moiety with the 2'-deoxyribosyl group linked to the phosphonate at either the 5'- or 3'-position and then added the thymine ring. Since the structure contained only two (3'-acylation) or three (5'-acylation) rotatable bonds, a systematic search identified the catalytically productive conformations (see Supporting Information). The shape of the nucleophile-binding region below the catalytic triad (medium pocket and alternate hydrophobic pocket) restricted the nucleoside orientations, especially the thymine ring orientation so that only a few conformations: a) contained all five catalytically essential hydrogen bonds (a-e) shown in Scheme 3A; b) avoided steric hindrances between the nucleoside phosphonate and the lipase; and c) avoided internal steric clashes within the nucleoside phosphonate. Acylation at the 5'-position allows rotation of three bonds in the nucleoside to adjust its position, while acylation at the 3'-position allows only two bonds to adjust. For this reason, modeling the 5'-butanoylation (PCL-5'-A-G) yielded a larger number of productive conformations than the 3'-butanoylation (PCL-3'-A-B), Table 2 and Table S1 in Supporting Information.

[Insert Scheme3]

[Insert Table2]

3' butanoylation catalyzed by PCL (favored)

The proposed productive conformation for butanoylation at the 3'-hydroxyl of thymidine is PCL-3'-A, which placed the thymine ring moiety in the alternate hydrophobic pocket and the 2'-deoxyribose moiety into the medium-sized pocket (Table 2, Figure 2A). Both moieties avoid steric hindrances in their respective pockets. Importantly, in addition to the five catalytically key hydrogen bonds, this intermediate formed three additional hydrogen bonds between thymine and the alternate hydrophobic

pocket (Figure 2B): from the 5'-OH to the backbone carbonyl oxygen of Leu17 (bond f, 2.81 Å, 153°),[21] from the phenolic hydroxyl of Tyr29 to the oxygen of the *O*-2 carbonyl of thymine (bond g, 3.04 Å, 168°), and between the *N*-3 of thymine and the phenolic OH of Tyr23 (bond h, 3.03 Å, 153°). The hydrophobic portion of the thymine (*C*5-*C*6-*C*7, Scheme 1) contacted the hydrophobic side chain of Leu287 (avg. C-C distance = 4.03 Å). We propose that these stabilizing hydrogen bonds and hydrophobic interactions cause PCL to favor acylation at the 3'-hydroxyl.

Conformation PCL-3'-B also formed all catalytically essential hydrogen bonds and avoided steric problems (Table 2). However, this conformation placed the thymine ring into solvent and therefore lacked the favorable interactions between the thymine moiety and the alternate hydrophobic pocket identified for conformation PCL-3'-A. For this reason, we expect the reaction to proceed via conformation PCL-3'-A, not PCL-3'-B.

[Insert Figure2]

5' butanoylation catalyzed by PCL (not favored)

The proposed productive conformation for butanoylation at the 5'-hydroxyl group is PCL-5'-A (Figure 2C) because it showed no obvious unfavorable steric interactions and maintained all five key hydrogen bonds. The thymine ring remained in the solvent, with the hydrophilic side of the thymine (*C*2-*N*3-*C*4) pointing to the solvent, and the hydrophobic side pointing into the large hydrophobic pocket, which contains the acyl chain. Thus, *C*7 of thymine is close to the propyl acyl chain (avg. *C*7-C_{propyl} distance = 4.35 Å). The hydrophilic oxygens of the 2'-deoxyribose showed minor unfavorable contacts with the hydrophobic groups in the active site. The 3'-OH of the 2'-deoxyribose lay close to the side chains of His286 (3.64 Å), Leu287 (3.60 Å), and Ile290 (3.24 Å), and the 2'-deoxyribose ring oxygen lay close to the side chain of Leu17 (3.15-4.03 Å).

Searching for additional conformations using molecular dynamics (see Supporting Information) yielded six similar conformations. Four models (PCL-5'-B to PCL-5'-E) showed only minor differences

from conformer PCL-5'-A, discussed above. Two models (PCL-5'-F and PCL-5'-G) showed a slightly different substrate and catalytic histidine orientations, but still had their thymine ring unbound in the solvent (Figure S2 in Supporting Information). Thus, substrate conformations for butanoylation at the 5'-hydroxyl position fit well in the active site of PCL, but did not make additional favorable interactions (*e.g.*, hydrogen bond or hydrophobic interaction) with the substrate-binding site. Attempts to place the thymine ring into the alternate hydrophobic subsite caused obvious steric contacts with Ile290 or Tyr29 or loss of catalytically relevant hydrogen bonds, while attempts to place it in the large hydrophobic pocket caused unfavorable intramolecular substrate interactions.

Discussion

Molecular modeling of phosphonate analogs of the tetrahedral intermediate suggests a molecular basis of the unusual regioselectivity of PCL for the more hindered 3'-alcohol in β-D-thymidine. During butanoylation of the 3'-hydroxyl, the thymine ring fits in an alternate hydrophobic pocket below the catalytic triad of the enzyme. Binding of the thymine base in this pocket involves both hydrophobic interactions and three extra hydrogen bonds. Models for butanoylation of the 5'-hydroxyl lack these interactions.

Other experimental observations support this hypothesis. Lipase from porcine pancreas (PPL) contains a similar alternate hydrophobic pocket below its catalytic triad and also favors 3'-acylation of β-D-thymidine.[12f,22] On the other hand, CAL-B lacks an alternate hydrophobic pocket and does not favor the 3'-position, but the 5'-position.[23] The orientation of the thymine ring is critical for it to fit into the alternate hydrophobic pocket during acylation of the 3'-hydroxyl. Stereoisomers of β-D-thymidine – α-D-thymidine and β-L-thymidine (Scheme 1) – differ in the orientation of the thymine ring. PCL does not favor acylation of the 3'-hydroxyl in these stereoisomers,[12e] but favors acylation of the 5'-hydroxyl.[16a]

Our hypothesis for β-D-thymidine may also apply to other β-D-2'-deoxynucleosides. Indeed, PCL-C catalyzes the 3'-regioselective acylations of other pyrimidine and purine nucleosides.[12a,12d,12f,16]

Although the structure of the bases differs from thymine and is larger for the purine nucleosides, all contain several heteroatoms and carbonyl groups that may afford similar stabilizing interactions in the hydrophobic pocket.

The modeling shows that the bond-making and bond-breaking parts of the tetrahedral intermediate are similar for both the 3'- and the 5'-acylation. Both contain all catalytically essential hydrogen bonds and avoid unfavorable steric interactions. The main difference is a better binding of the 3'-intermediate at a site remote from the reaction center. Such remote binding can lower the free energy of the corresponding transition state by favoring a catalytically productive orientation.[24] Holding reactive groups in an orientation that favors reaction can accelerate reaction more than a million-fold as discussed by A. J. Kirby (high effective molarity[25]) and T. C. Bruice (formation of a near-attack complex[26]). Since a hydrogen bond between neutral partners contributes ~1 kcal/mol to binding, the three hydrogen bonds between thymine and PCL may contribute ~3 kcal/mol (or >100-fold rate acceleration) to the preference acylation for the secondary hydroxyl. Due to dynamic effects in proteins it can be difficult or impossible to dissect enzyme-substrate interactions into purely ground state binding *vs*. purely transition state binding contributions.[27]

This hypothesis for the origin of the preference for the 3'-hydroxyl may be extended to also rationalize the higher regioselectivity with larger acyl groups. The selectivity for the 3'-hydroxyl is lowest for acetylation (Table 1, entry 1, 1.3:1), higher for butanoylation (entry 2, 6.9:1), and even higher for decanoylation (entry 3, >98:1), for benzoylation (entry 4, >24:1), and for phenylacetylation (entry 6, >42:1). This increased regioselectivity may be due to a destabilization of the conformation PCL-5'-A, which forms the minor regioisomer, by the larger acyl group. The *C*7 of thymine points into the large hydrophobic pocket, which also binds the acyl group (avg. $C7$-C_{propyl} distance = 4.35 Å). A larger acyl group may create steric strain, destabilize this conformation, and decrease the amount of the minor regioisomer.

Previous modeling studies also attributed the molecular basis of enzyme regioselectivity to favorable interactions remote from the reaction site. For example, Botta *et al.*[28] proposed that the high

regioselectivity of *Mucor miehei* lipase toward *C*-2 side chain hydroxyl group on resorcin[4]arenes stems from a favorable π-stacking between a phenyl ring of the substrate and Trp88, (~11 Å from the catalytic serine) and a hydrogen bond between *C*-14 side chain hydroxyl group with Asp91 in the favored isomer. Gascoyne and coworkers[29] proposed that the high regio- and enantioselectivity of *Chromobacterium viscosum* lipase (CVL) in the hydrolysis of diesters derived from 2,3-dihydro-3-(4'-hydroxyphenyl)-1,1,3-trimethyl-1H-inden-5-ol stems from a remote hydrophobic interaction between the 4'-acyl group and the large hydrophobic pocket. In a third, example, the high regioselectivity of CAL-B for the primary 5'-hydroxyl of thymidine was attributed to a remote hydrophobic interaction between the thymine ring and the large hydrophobic pocket. Binding of the 3'-hydroxyl in a reactive orientation placed the thymine ring in the solvent where it could not make favorable contacts with the enzyme.[23]

However, favorable interactions at a remote site are not always the explanation for regioselectivity. In one case, Borreguero *et al.*[30] proposed that favorable interactions *close to* the reactive site accounts for the regioselectivity. The high regioselectivity of porcine pancreatic lipase for the primary alcohol of phenylalkanediols stems from a hydrogen bond between the secondary hydroxyl and catalytic histidine and a π-stacking interaction with the side chain of a phenylalanine (Phe216) close to the catalytic serine.

In another case, Rich *et al*.[31] suggested that *unfavorable* interactions for the slow regioisomer account for the selectivity. They suggested that subtilisin favors acylation of the 1'-hydroxyl over the 6-hydroxyl of sucrose because the alcohol-binding pocket hinders the 6-hydroxyl reactive orientation. Similarly, they suggested[31] that the poor regioselectivity of subtilisin toward thymidine was due to similar orientations of both regioisomers in the alcohol binding site.

Chemical reagents are small compared to enzymes and thus, cannot use remote interactions to influence enantio- or regioselectivity. The higher selectivity of enzymes likely stems from their ability to interact with larger portions of the substrate. The regioselective lipase-catalyzed acylation of nucleosides enables modification of non-natural nucleoside analogs,[33] use of acyl groups as protecting groups during nucleoside synthesis,[34] and preparation of more bioactive nucleoside derivatives.[35] The

importance of remote interactions for this regioselectivity suggest that many other examples of enzyme-catalyzed selective modification of complex molecules await discovery.

Experimental Section

Supporting Information contains details on the PCL-C-catalyzed acylations of thymidine (data shown in Table 1) and the molecular modeling. The program Insight II, version 2000.1, was used for viewing the structures. The geometric optimizations and molecular dynamics were performed using Discover, version 2.9.7 (*Accelrys*, San Diego CA, USA), using the AMBER[36] force field. The distance dependent dielectric constant was set to 4.0 to mimic the electrostatic shielding of the solvent and the 1-4 van der Waals interactions were scaled to 50%. The crystal structure for the PCL (3LIP[17]) was obtained from the Protein Data Bank (www.rcsb.org/pdb/), and includes a phosphonate. Protein structures in Figures 1 and 2 were generated using PyMOL 0.97.[37]

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Supporting Information Available. Supporting information contains molecular modeling details and experimental procedures, including ^{1}H- and ^{13}C-NMR spectra, melting points, infrared spectra, microanalysis, and mass spectrometry data. This material is available free of charge via the internet at http://www.chembiochem.org.

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Legends for figures and schemes

Scheme 1. Regioselectivity of PCL-C (*Pseudomonas cepacia* lipase immobilized on ceramic beads) or CAL-B (*Candida antarctica* lipase B) toward several stereoisomers of thymidine. In most cases these lipases favor acylation of the less-hindered 5’-hydroxyl, but for β-D-thymidine, PCL-C favors the more-hindered 3’-hydroxyl.

Scheme 2. Regioselective PCL-C-catalyzed acylation of thymidine (**1**, T= thymine).

Scheme 3. Tetrahedral intermediate for butanoylation of ethanol and the corresponding phosphonate analog. A) The five catalytically essential hydrogen bonds are: one from the carboxylate of Asp264 to N_δ of His286 (bond a), two from N_ε of His286 to the oxygens of Ser87 (bond b) and the ethoxy group (bond c), and two from the oxyanion to the main chain amides of Gln88 (bond d) and Leu17 (bond e). B) A phosphonate analog mimicked the tetrahedral intermediates in computer-aided molecular modeling. To model the butanoylation of thymidine, the ethoxy group of the phosphonate was replaced by either the 3'- or 5'-OH of the thymidine as described in the text.

Figure 1. PCL active-site structure from X-ray crystallography (pdb code: 3LIP[17]) viewed with the catalytic triad Asp264-His286-Ser87 oriented from left to right and shown in sticks representation (Asp264 is hidden). The acyl group of the substrate usually binds in the large hydrophobic pocket (green spacefill) above the catalytic triad. This arrangement places the leaving-group alcohol of the nucleoside below the catalytic triad in the medium-sized pocket with the rest of the nucleoside extending into the solvent or the alternate hydrophobic pocket (dark blue spacefill). The backbone amide groups of Gln88 (yellow spacefill) and Leu17 (red spacefill) form the oxyanion hole and contribute to catalysis by stabilizing the oxyanion of the tetrahedral intermediate.

Figure 2. Best models of phosphonate analogs for PCL-catalyzed butanoylation of thymidine. Residues surrounding the large hydrophobic pocket (Val266, Val267, Leu167, Phe119 and Pro113) are colored green and residues surrounding the alternate hydrophobic pocket (Ile290, Leu287, Thr18 and Tyr29) are colored dark blue. Oxyanion hole residues Leu17 and Gln88 are colored red and yellow, respectively. (A) Conformation PCL-3'-A of the phosphonate mimics butanoylation of the 3'-hydroxyl and places the thymine ring in the alternate hydrophobic pocket. (B) When placed in this alternate pocket, the thymine

formed three additional hydrogen bonds (bonds f-h). We hypothesize that this tighter binding of the thymine ring to the active site is the molecular basis for the preferential acylation of 3'-hydroxyl of thymidine by PCL-C. (C) Conformation PCL-5'-A mimics butanoylation of the 5'-hydroxyl and showed an unbound thymine ring pointing toward the solvent with its hydrophobic side close to the propyl acyl chain (see the text). To allow a better view of the active site, Figures A and C display Ala247 and Leu248 in a line representation.

Tables

Table 1. Regioselectivity of PCL-C-catalyzed acylation of thymidine with different oxime esters.[a]

Entry	acyl group	R	temp (ºC)	*t* (h)[b]	conv (%)	3' (%)	5' (%)	3',5' (%)	3':5'-regio-selectivity[c]
1	acetyl	Me	30	1	92	44	30	18	1.3:1
2	butanoyl	Pr	30	1.6	92	79	2	11	6.9:1
3	decanoyl	Non	30	2.8	98	98	0	0	>98:1
4	benzoyl	Ph	30	39	24	24	0	0	>24:1
5	benzoyl	Ph	60	22	54	48	6	0	8:1
6	phenylacetyl	Bn	30	37	42	42	0	0	>42:1
7	phenylacetyl	Bn	60	22	75	70	5	0	14:1

[a] The lyophilized protein (unimmobilized) also catalyzed these reactions with similar selectivities (data not shown), but the rate was much slower. Reactions were monitored by gas chromatography (GC), except for the acetylation reaction. In this case, peaks of the acetylation products overlapped on GC chromatograms, so reaction was monitored by HPLC. [b] Time refers to either the time to reach high conversion or the time where no further reaction was observed. [c] Selectivity was estimated from the ratio of the 3'- and 5'-monoacylated products. Diacylated compounds include both a 3'- and a 5'-acylation, so the amount of diacylated derivative was added to each of the 3'- and 5'-monoacylated products to estimate the selectivity.

Table 2. Geometry-optimized conformations of phosphonate analogs of tetrahedral intermediates for butanoylation of thymidine catalyzed by PCL obtained by molecular modeling and molecular dynamics.[a]

Regioisomer	Structure	Key H-Bonds	Thymine Ring Binding
3’	**PCL-3’-A**	5 of 5	Alternate Pocket
3’	PCL-3’-B	5 of 5	Un-bound
5’	**PCL-5’-A**	5 of 5	Un-bound
5’	PCL-5’-F[b]	5 of 5	Un-bound

[a] All models are derived from the phosphonate tetrahedral intermediate analog for butanoylation of ethanol. Hydrogen bonds are those with N–O distances of <3.2 Å and N–H–O angles >120°. Proposed productive conformations are in bold. [b] See Supporting Information for details.

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