Controlling Subtilisin Activity and Selectivity in Organic Media by Imprinting with Nucleophilic Substrates

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Abstract: The activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the enzyme from an aqueous solution containing the substrate. This "molecular imprinting" technique was examined using thymidine as a model nucleoside, and the resulting subtilisin preparation was up to 50-fold more reactive toward thymidine acylation in nearly anhydrous tetrahydrofuran than subtilisin lyophilized from aqueous buffer in the absence of the nucleoside. Although several compounds lyophilized with subtilisin, including thymine and ribose, improved the rate of thymidine acylation, the thymidine-imprinted enzyme was the most efficient catalyst for this reaction. Furthermore, it was possible to alter the substrate selectivity of subtilisin by lyophilizing the enzyme in the presence of a different nucleophilic substrate. For example, imprinting made possible the discrimination between structurally different (i.e., sucrose versus thymidine) as well as structurally similar (i.e., thymidine versus deoxyadenosine) nucleophiles. Molecular modeling studies of the interaction of thymidine or the unrelated sucrose with subtilisin revealed that structural changes upon imprinting in the serine protease's catalytic triad may be responsible for the observed activation and selectivity changes. Further use of molecular dynamics indicated that structural changes in the catalytic triad occur during imprinting, and that these changes may be the major factor that contributes to imprinting-induced substrate selectivity. This contrasts with the previously held notion that imprinting influences mainly substrate binding.

Introduction

The regioselective modification of polyfunctional compounds, including sugars and their derivatives, remains an arduous, yet important, task in organic chemistry. The selective acylation of nucleosides, for example, is important in the development of new methods for the synthesis of nucleoside analogs. Among their varied biological effects, nucleoside derivatives have been shown to possess antiviral[1] and antineoplastic[2] activities. The acylation of nucleosides has traditionally involved blocking−deblocking methodologies or the preferential modification of the primary groups (5′-OH) by reaction with bulky acylating agents which are sterically hindered from reacting at secondary positions.[3] Enzymes in aqueous solution have been effective in the regioselective hydrolysis of nucleoside di-[4] or triesters.[5] Alternatively, enzymes suspended in a suitable organic solvent (e.g., pyridine or *N*,*N*-dimethlyformamide for carbohydrates and tetrahydrofuran (THF) or *tert*-amyl alcohol for nucleosides) are capable of catalyzing the regioselective acylation of primary[6] and secondary hydroxyl[7] groups of sugars and nucleosides.[8]

Although exquisite selectivity has long been a hallmark of enzymatic catalysis, enzymes have only been weakly active in organic solvents as compared with their "native" activities in water.[9] To expand the application of enzymes as catalysts for synthetic reactions in organic media, it is necessary to overcome this diminished activity upon suspension in anhydrous or nearly anhydrous solvents.[9,10] Furthermore, the control of enzyme specificity (e.g., substrate specificity, enantio-, chemo-, and regioselectivity) has become an increasingly important goal in enzyme technology. To that end, alteration of the protein primary structure, via site-specific[11] and random[12] mutagenesis techniques, and the conservation of the secondary structure via the use of lyoprotectants (e.g., carbohydrates, inorganic salts)[13]

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have been used to increase enzyme activity and stability in organic solvents, as well as to tailor reaction selectivity. In particular, subtilisin Carlsberg imprinted with competitive inhibitors (via lyophilization of the enzyme from an aqueous solution containing the inhibitor)[14] and substrate analogs[13a] shows increased rates of transesterification of various amino acid esters up to 365-fold over the nonimprinted enzyme.[15]

The use of additives to the aqueous solution prior to freezing has been shown to prevent the reversible denaturation of proteins during the drying process.[16] For example, lyoprotectants, including sucrose, trehalose, and other carbohydrates, and polymers such as poly(ethylene glycol), have been shown to increase the activity of *Aspergillus oryzae* protease suspended in anhydrous organic solvents (including pyridine and carbon tetrachloride) in the transesterification of *N*-acetyl-L-Phe-OEtCl with 1-propanol or vinyl butyrate with benzyl alcohol.[13a] In previous reports we showed that lyophilizing subtilisin with a 2-fold weight excess of sucrose increased catalytic performance up to 60-fold for the acylation of sucrose with vinyl esters in pyridine.[6b,17] In the present study, we have extended the application of molecular imprinting to include the activation of enzymes for use in organic solvents through imprinting with other nucleophilic subtstrates (e.g., nucleosides), including the first report of reversing the substrate specificity of subtilisin. The results described herein indicate that molecular imprinting can be an effective means toward achieving transition state stabilization and alteration of enzyme selectivity.

Materials and Methods

Enzymes. Subtilisin Carlsberg (alkaline serine protease from *Bacillus licheniformis*) was purchased from Sigma (St. Louis, MO). Lipase PS30 was purchased from Amano (Troy, VA) and was used without purification or modification. Semipurified subtilisin BPN′ (alkaline serine protease from *Bacillus amyloliquefaciens*) wild-type and mutant enzymes were generously provided by Thomas Graycar at Genencor International. All BPN's were purified as described previously.[18]

Imprinting of Enzymes. A typical preparation of enzymes for imprinting involved dissolving 20 mg of purified enyzme in 2 mL of 10 mM phosphate buffer (pH 7.8) containing 40 mg of nucleophilic substrate (e.g., thymidine). Native subtilisin was prepared by dissolving 20 mg of subtilisin in 2 mL of 10 mM phospate buffer (pH 7.8).[19] The solution was then flash-frozen in liquid N_2 and lyophilized on a Labconco (Kansas City, MO) Lyph-Lock-12 freeze dryer for 24 h. Lyophilized enzyme preparations were stored in a desiccated (Drierite) environment at −20 °C.

Solvents and Chemicals. Vinyl butyrate was purchased from TCI America (Portland, OR). All solvents were obtained from commercial suppliers and were of the highest purity available. The solvents were stored under N_2 atmosphere over 3 Å molecular sieves (Linde) and had a water content of <0.005% (w/v) as determined by the limit of Karl-Fischer titration. Analytical grade solvents for thin layer and flash column chromatography were used without further purification. Nucleosides (deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG), and thymidine), carbohydrates (sucrose and ribose), and bases (thymine and adenine) were purchased from Sigma.

Small Scale Reactions. A typical nucleoside acylation experiment involved suspending 1 mg of enzyme preparation in 10 mL of anhydrous organic solvent containing 10 mM nucleoside and 100 mM vinyl butyrate. The mixture was shaken (300 rpm) at 30 °C with 40 μL aliquots periodically removed for analysis. Sucrose acylation reactions were performed as described previously.[6b]

Analytical Techniques. The progress of nucleoside ester synthesis was followed by reversed-phase HPLC (Shimadzu 10LC) using a YMC ODS-AQ (4 mm × 25 cm) column with an isocratic mobile phase, for thymidine, of 60% water and 40% acetonitrile or, for other nucleosides, of 90% water and 10% acetonitrile. UV detection was at 254 nm (thymidine, dA, dG) or 280 nm (dC). In all cases the order of elution was unreacted nucleoside, nucleoside 5′-monoester, and nucleoside 3′-monoester.[4a] Sucrose acylation reactions were followed on a 10 m AT-1 capillary (Alltech) column on an HP-5890A gas chromatograph equipped with a flame-ionization dectector. All samples were precolumn derivitized with 1,1,1,3,3,3-hexamethyldisilazane (Sigma Sil-A).

Determination of Product Structure. The Lipase PS-prepared thymidine 3′-butyrate product was an amorphous solid: ^{13}C NMR (Brücker WM 360 MHz, DMSO-d_6, TMS as the internal reference) δ 172.4, 163.6, 150.5, 135.8, 109.7, 84.7, 83.7, 74.6, 61.3, 36.5, 35.3, 17.8, 13.3, 12.2. The subtilisin Carlsberg-prepared thymidine 5′-butyrate product was also an amorphous solid: ^{13}C NMR (DMSO-d_6, TMS as the internal reference) δ 172.6, 163.6, 150.4, 135.8, 109.7, 83.8, 83.6, 70.2, 63.7, 38.6, 35.2, 17.9, 13.3, 12.1. The subtilisin-prepared deoxyadenosine 5′-butyrate product was an amorphous solid: ^{13}C NMR (DMSO-d_6, TMS as the internal reference) δ 172.6, 156.1, 156.1, 149.2, 139.5, 119.3, 83.6, 81.7, 74.2, 63.4, 38.8, 35.4, 17.8, 13.4. The lipase-prepared deoxycytidine 3′-acrylate product was an amorphous solid: ^{13}C NMR (DMSO-d_6, TMS as the internal reference) δ 165.5, 164.8, 155.9, 139.0, 132.2, 127.9, 102.1, 84.8, 84.5, 75.2, 61.3, 28.9. The subtilisin-prepared deoxyguanosine 5′-butyrate product was also an amorphous solid: ^{13}C NMR (DMSO-d_6, TMS as the internal reference) δ 170.0, 162.0, 139.1, 124.8, 124.8, 76.8, 72.4, 69.3, 62.9, 62.9, 35.8, 35.5, 17.9, 13.5. Following the strategy of previous ^{13}C NMR analyses of sugars,[20] acylation at position 5′ of ribose should result in a downfield shift of the peak at C5′ and an upfield shift of the peak at C4′. Acylation at position 3′ of ribose should result in a downfield shift of the peak at C3′ and an upfield shift in the peaks at C2′ and C4′. These shifts are virtually independent of the nature of the acyl moiety and the solvent.[17,21] Comparison of the NMR spectra to that of thymidine indicates an upfield shift of the C4′ and downfield shift of the C5′ for the subtilisin Carlsberg-prepared product and an upfield shift of the C2′ and C4′ and downfield shift for the C3′ for the Lipase PS-prepared product. The NMR results are consistent with deoxynucleoside esters produced enzymatically by others.[8b] This strongly suggests that subtilisin Carlsberg and Lipase PS catalyze the preferential synthesis of thymidine 5′-butyrate and thymidine 3′-butyrate, respectively.[4b,8b]

Conformational Analysis. Construction of the molecular model of the thymidine tetrahedral intermediates was performed following our previous methodology.[6b] All models were evaluated on a Silicon Graphics Indigo^2 XZ workstation equipped with Sybyl 6.2.[22] After construction of the tetrahedral intermediate, the resulting bond (Ser_{221}−O−C(R)−O$^-$) was rotated such that at least part of thymidine occupied the binding pocket. The evaluation of possible conformations was accomplished using the Systematic Search feature of Sybyl with a

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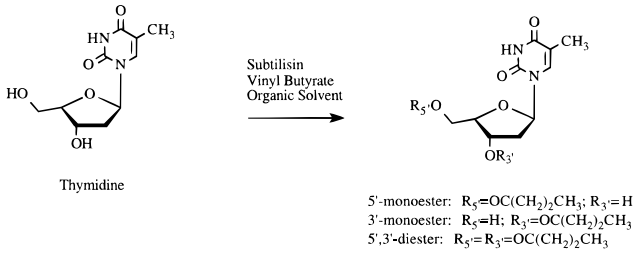
Scheme 1. Reaction Scheme of the Subtilisin-Catalyzed Acylation of Thymidine with Vinyl Butyrate



General Scale factor of 0.30 and rotation about the tetrahedral intermediate bond (for both sucrose and thymidine) and the aminoglycosidic bond (for thymidine). The ratio of the total available realistic conformations (total conformations less trivial conformations) for each monoester intermediate was related to the actual observed regioselectivity for the pH-adjusted enzyme. This provided a direct comparison of the observed regioselectivity with our developed model. The Pullman charge calculation method has been shown to be effective in the calculation of dipole[23] and was employed to calculate the dipole moment of thymidine. Molecular dynamics involved the equilibration of the model at 0 K for 1 ps (1000 time steps) followed by heating the molecule to 100 K (linear gradient over 100 fs) and simulation at 100 K for 10 ps (10 000 time steps). The conformation of the enzyme was stored every 50 time steps. The Amber-United force field and charge set were used for all simulations. The nucleophilic substrate molecules and Ser_{221} were constrained using the SHAKE algorithm; no constraints were placed on any enzyme residues.

Results and Discussion

We have previously shown that imprinting substilisin (BPN′ and Carlsberg) with sucrose resulted in a rate enhancement for acylation with vinyl esters in organic solvents.[6b] The imprinting methodology has been extended in this work to the subtilisin-catalyzed acylation of nucleosides. The simple pyrimidine deoxynucleoside, thymidine, has been chosen as a model substrate for this study, and acylation can occur at the 5′- and 3′-hydroxyl groups (Scheme 1). Subtilisin Carlsberg preferentially acylates thymidine in THF at the 5′-hydroxyl (thymidine 5′-butryate/thymidine 3′-butyrate = 2.15) using vinyl butyrate as acyl donor.[24] Such moderate regioselectivity was not significantly dependent on the nature of the organic solvent with regioselectivities of 5-butyrylation/3′-butyrylation of 2.15−1.99 in solvents ranging from the highly polar (THF) to the moderately nonpolar (*tert*-amyl alcohol). It should be noted that thymidine is not significantly soluble in solvents more hydrophobic than *tert*-amyl alcohol.

Thymidine Acylation. The effectiveness of thymidine as a molecular imprint on subtilisin Carlsberg-catalyzed acylation of thymidine with vinyl butyrate was dependent on the concentration of the nucleoside in the aqueous solution prior to lyophilization (Figure 1). A rate enhancement of ca. 50-fold occurred at a thymidine concentration of 1% (w/v) (a weight ratio of 1:1 thymidine:enzyme) or ca. 100-fold molar excess of thymidine in the lyophilizate; no further increase in rate enhancement was evident above this weight ratio. Subsequent experiments were performed with subtilisin lyophilized from a 2% (w/v) excipient solution. This activation is not due to an increase in the local concentration of thymidine as the nucleoside is soluble in THF and dissociates from the insoluble enzyme

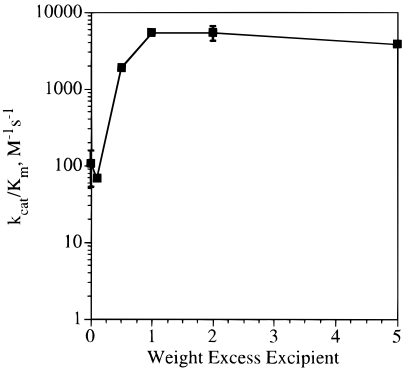


Figure 1. Dependence of the imprinting-induced rate enhancement on excipient concentration in the lyophilizate. Various amounts (expressed in weight excess of excipient compared to enzyme) of thymidine were dissolved in 10 mM sodium phosphate buffer (pH 7.8) containing 20 mg of subtilisin (10 mg of enzyme/mL of buffer). This solution was then flash frozen in liquid N_2 and lyophilized for 24 h. A 2-fold weight excess of thymidine corresponds to a ca. 200-fold molar excess. Error bars represent ±1 standard deviation for zero-fold (N=3) and two-fold (N=4) excess thymidine.

preparation. Indeed, thymidine-imprinted subtilisin Carlsberg did not catalyze the acylation of benzyl alcohol with vinyl butyrate in hexane (thymidine is not soluble in hexane), whereas the native enzyme was an efficient catalyst for this reaction. Therefore, an inactive enzyme preparation results when the imprinting molecule is not soluble in the reaction medium, thereby presumably blocking the active site. Moreover, addition of 100-fold molar excess of thymidine to native subtilisin Carlsberg suspended in THF followed by replacement of the solvent with fresh THF, containing 10 mM thymidine and 100 mM vinyl butyrate, did not result in rate enhancement over native subtilisin.

These results suggest that the process of *lyophilizing* subtilisin in the presence of thymidine leads to an activated enzyme preparation, and that this enzyme preparation becomes catalytic upon dissociation of the thymidine from the lyophilizate in a suitable organic solvent. Furthermore, the lack of activation upon pretreating the suspended enzyme preparation with the imprint molecule in THF suggests that a property of the enzyme in THF prevents imprinting from being effective. Such a property may be flexibility, and the known decrease in protein flexibility in organic solvents as compared to water[25] may prevent the enzyme molecule from incorporating the imprint molecule into its active site and molding its active site structure around the imprint molecule. The high reactivity of the thymidine-imprinted subtilisin Carlsberg (k_{cat}/K_m = 5390 M^{-1} s^{-1}) is also reflected in high conversion yields. For example, in the presence of only 30 μg/mL enzyme, nearly full conversion of 10 mM thymidine to thymidine butyrate was obtained in less than 1 h.

To determine if only a part of thymidine was responsible for the observed rate enhancement, subtilisin Carlsberg was lyophilized in the presence of thymine and ribose (2′-deoxyribose was poorly soluble in THF) for use as catalysts in the acylation of thymidine with vinyl butyrate in THF (Table 1). Both the base and the sugar were capable of activating subtilisin for thymidine acylation, albeit to a lesser extent than by imprinting with thymidine itself. Interestingly, the imprinting-induced rate enhancement was due to an increase in k_{cat}, with only minimal alteration in K_m (Table 1). Thus, the imprinting phenomenon appears to be fairly selective in its effect, and strongly affects

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(24) The 5′-butyrate was synthesized using subtilisin Carlsberg to generate both the 5′- and 3′-esters followed by silica gel flash column chromatography purification of the 5′-ester. The 3′-ester was synthesized using Lipase PS30 and purified by flash column silica gel chromatography. Similar reactions and purifications were performed with the other deoxynucleosides employed in this study.

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Table 1. Rate Enhancement of the Acylation of Thymidine with Vinyl Butyrate in THF Catalyzed by Different Subtilisin Carlsberg Preparations[a]

excipient	k_{cat}/K_m, $M^{-1}\ s^{-1}$	k_{cat}, s^{-1}	K_m, mM	rate enhancement
none	104	0.694	6.66	1.00
ribose	2090	5.90	2.83	20.1
thymine	2870	13.3	4.63	27.6
thymidine	5390	26.5	4.93	51.8

[a] The concentration of vinyl butyrate was 100 mM, the concentration range of thymidine was 0.5−20 mM, and the concentration of the enzyme preparation was 0.1 mg/mL (containing 0.033 mg/mL subtilisin). The reactions were shaken at 300 rpm at 30 °C.

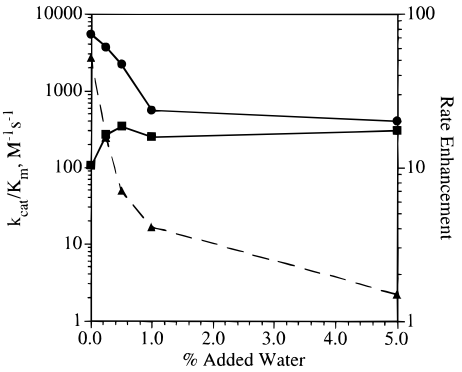

Figure 2. Magnitude of the imprinting-induced rate enhancment is controlled by enzyme hydration. Upon the addition of 5% (v/v) water to the organic solvent, the imprinted enzyme has the same activity as the pH-adjusted enzyme. Thymidine acylation in THF catalyzed by thymidine-imprinted (●) and native (■) subtilisin Carlsberg. The imprinting-induced rate enhancement (▲) is shown by the broken line. All enzyme preparations contained a 2-fold excess of excipient. Two phenomena occur: (i) a decrease in the activity of the imprinted enzyme and (ii) an increase in the activity of the pH-adjusted (nonimprinted) enzyme.

the catalytic components of enzyme activity as compared to binding components.

Effect of Water Addition on Imprinting. The activation phenomenon was strongly depressed upon addition of water to the organic solvent (Figure 2). Specifically, the rate enhancement for thymidine acylation dropped from 52-fold in anhydrous THF to 1.48 in THF containing 5% (v/v) water. Interestingly, the catalytic efficiency of the native enzyme is *increased* 3-fold to ca. 300 $M^{-1}\ s^{-1}$ by adding 5% water to THF. The activation of the native enzyme upon water addition is not surprising; a small concentration of water is known to activate enzyme preparations, albeit only modestly.[26,27] Conversely, the thymidine-imprinted enzyme was *deactivated* by the addition of small amounts of water to the organic solvent; the catalytic efficiency of the thymidine-activated subtilisin Carlsberg dropped from 5390 to ca. 400 $M^{-1}\ s^{-1}$ in THF containing 5% (v/v) water. Thus, the decreased rate enhancement for thymidine acylation upon addition of 5% (v/v) water was the result of a rate *increase* (ca. 3-fold) of the native enzyme and a rate *decrease* (ca. 15-fold) of the thymidine-imprinted enzyme.

Why does water strongly suppress imprinting-induced activation? Such a phenomenon has been observed previously; the activiation of subtilisin Carlsberg by imprinting with a competitive inhibitor was lost by the addition of 0.4% water to octane.[14] For imprinting to improve enzymic catalytic performance, we may speculate that it is necessary for the excipient (imprint) to "mold" the enzyme into a catalytically more favorable conformation.[14] We may further speculate that increased water content in THF results in a hydrated enzyme preparation with greater overall flexibility, particularly at the active site of the enzyme.[27] This plasticizing effect of water may loosen the structure of the enzyme's active site, thereby relaxing the forces that govern enzyme−imprint interactions and negating some of the activating effect of the imprint. This hypothesis has some precedence. Specifically, enzyme flexibility has been shown to increase, via fluorescence anisotropy[28] and ESR[27] studies, upon the addition of small concentrations of water. Therefore, the increased flexibility allows the enzyme to effectively "shake off" the excipient-induced conformational changes.

Table 2. Relative Rate Enhancement[a] of Several Mutants in the S_1' Pocket of Subtilisin BPN′ for the Acylation of Thymidine with Vinyl Butyrate in THF

enzyme	relative rate enhancement[a]	enzyme	relative rate enhancement[a]
wild type	1.00	M222F	0.99
Y217L	0.88	M222K	0.04
M222A	1.04	M222Q	0.87
M222D	0.54		

[a] Relative rate enhancement (RRE) = $(v_{imprint}/v_{native})_{mutant}/(v_{imprint}/v_{native})_{wild\ type}$. All other conditions as described in the legend to Table 1.

Imprinting of Protein-Engineered Subtilisins. The imprinting phenomenon was further probed by site-directed mutagenesis. To that end, we examined the effect of thymidine imprinting on the related enzyme, subtilisin BPN′. Wild-type and mutant enzymes, containing a single site-specific change in the S_1' binding pocket of enzyme, were used. The effectiveness of protein engineering on imprinting was measured by comparing the rate enhancment of the mutant enzymes to that of the wild-type enzyme (Table 2). The ratio of rate enhancements (relative rate enhancement (RRE)) provides a direct measure of the ability of protein engineering to alter the interaction of the imprinter with the enzyme and normalizes the result to the baseline rate enhancement of the wild-type enzyme. This comparison reveals that the effectiveness of imprinting is altered only for the M222D and M222K enzymes. Specifically, the rate enhancements of the Asp and Lys mutants are 54% and 4%, respectively, of the wild-type enzyme. Both of these mutations introduce an electrostatic charge (M222D has −1 charge and M222K has +1 charge) into the active site, thereby potentially disrupting the transition state stabilization of the tetrahedral intermediate.[29] The +1 charge on the Lys mutant side chain may strongly interact with the dipole moment of thymidine ($\mu \cong 5.7$ D[30]), resulting in a dramatically lower enzyme activity by preventing the substrate access to the formed acyl enzyme intermediate (similar to the imprint being insoluble in the reaction medium) as well as having an attractive interaction with the oxyanion formed during the tetrahedral intermediate of the deacylation step. While the Asp mutant (−1 charge) may not result in a blocked active site, it is speculated that the charge will have a repulsive interaction with the negatively charged oxyanion of the tetrahedral intermediate.

Although the Y217L and M222F enzymes did not alter the rate enhancement, these site-specific mutants were 2- and 3-fold more active, respectively, than the wild-type enzyme. Un-

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(30) The dipole moment for thymidine was calculated using the Pullman charge calculation method within SYBYL. The SYBYL command COMPUTE...DIPOLE was used to determine the dipole moment.

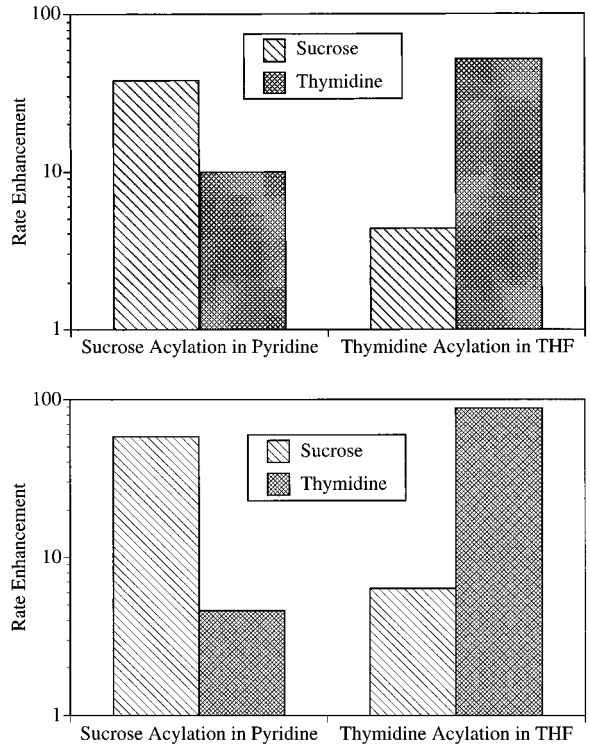

Figure 3. Enhancement of substrate specificity by imprinting subtilisin Carlsberg (A, top) and subtilisin BPN′ (B, bottom) with structurally different nucleophilic substrates. Sucrose- and thymidine-imprinted subtilisin Carlsberg were employed as catalysts for the transesterification of sucrose or thymidine with vinyl butyrate. Sucrose and thymidine acylation reactions were performed in pyridine and THF, respectively.

charged mutations to the S_1 pocket did not result in a significant change in the effectiveness of imprinting (Table 2). Replacement of Met_{222} with a smaller side chain (M222A), which resulted in a larger S_1 pocket, did not significantly alter the relative rate enhancement (RRE = 1.04). Furthermore, replacement of Met_{222} with Phe (hydrophobic ring structure) or Gln (amide side chain) and Tyr_{217} with Leu (branched hydrocarbon side chain) did not change significantly the ability of imprinting to improve the catalytic rate of subtilisin BPN′-catalyzed thymidine acylation.

Substrate Selectivity of Imprinted Enzymes. How structurally selective are enzymes imprinted with nucleophilic substrates? Initially, two structurally unrelated nucleophiles, sucrose and thymidine, were chosen as model nucleophiles and imprinters in subtilisin Carlsberg catalysis. Imprinting was performed with thymidine and sucrose separately. The two imprinted enzyme preparations were then examined for *both* sucrose and thymidine acylation. For sucrose acylation, the sucrose-activated and thymidine-activated enzymes were 40-fold and 4-fold more active, respectively, than the native enzyme (Figure 3A). However, the trend was reversed for thymidine acylation where the sucrose-activated enzyme and the thymidine-activated enzyme were 9-fold and 52-fold more active than the native enzyme. A strikingly similar result is found for the related subtilisin BPN′ (Figure 3B). Enzymic substrate specificity was, therefore, *inverted* by imprinting the enzyme with structurally different nucleophilic substrates.

The true indication of selectivity reversal can be measured by examining the selectivity of activated enzyme on an equimolar (10 mM) mixture of sucrose and thymidine. Significantly, the thymidine-activated subtilisin was ca. 6-fold more active toward the acylation of thymidine than was the sucrose-activated enzyme. Conversely, the sucrose-imprinted subtilisin was ca. 5-fold more reactive toward the acylation of sucrose than was the thymidine-activated enzyme. The measurable activation of subtilisin Carlsberg by the unmatched substrate−enzyme pairs (i.e., activation by sucrose for the thymidine-imprinted enzyme and activation by thymidine for the sucrose-imprinted enzyme) cannot be explained by imprinting selectivity. However, we cannot rule out the baseline effect of lyoprotection by an excipient that can interact with the active site of the enzyme. This hypothesis is made more compelling by the presence of nonreducing sugar moieties in the two substrates (sucrose itself and the deoxyribose sugar of the thymidine) and the ability of nonreducing sugars to act as effective lyoprotectants.[31]

Table 3. Alteration of Substrate Specificity by Imprinting Subtilisin Carlsberg with Structurally Similar Nucleophilic Substrates

	rate enhancement,[a] $v_{imprint}/v_{native}$			
excipient	thymidine[b]	dC[b]	dA[b]	dG[b]
thymidine	51.8	101	7.80	1.67
dC	40.0	182	20.6	3.89
dA	37.8	96	31.0	2.67
dG	9.61	58	2.80	4.78

[a] Rate enhancement is the ratio of the reaction rates (v) of the imprinted and native enzymes. Nucleoside-imprinted enzymes were employed as the catalyst for the acylation of like and different nucleosides. [b] Reaction conditions: thymidine (10 mM) in THF, dC (1 mM) in THF, dA (5 mM) in THF, and dG (1 mM) in THF containing 20% (v/v) DMF. All reactions contained vinyl butyrate (100 mM) as the acyl donor, and enzymes were prepared by lyophilizing subtilisin Carlsberg in the presence of a 2-fold weight excess of nucleoside. The bases of the nucleosides range in size from 80 Å^3 (dC) to 102 Å^3 (dG). Volumes were calculated by generation of a Connelly surface using a 1.4 Å probe radius.

The substrate specificity of the imprinting phenomenon was also evident for structurally similar nucleophiles. Specifically, subtilisin Carlsberg lyophilized in the presence of different nucleosides had observed rate enhancements for acylation of the matched substrates (e.g., thymidine imprinting yielded a subtilisin preparation with the highest activity on thymidine acylation, and dG imprinting yielded a subtilisin preparation with the highest activity on dG acylation, etc.) (Table 3). This activation phenomenon was observed for the acylation of both pyrimidines (thymidine, dC) and purines (dA, dG). Competition for reactivity by the imprint molecule versus the substrate (for cross-reactivity experiments) was minimized by using a small enzyme (and hence imprint) concentration in acylation reactions. The rate enhancement was dependent on the nucleoside, ranging from 182-fold for dC acylation to ca. 5-fold for dG acylation. It should be mentioned that the dG-activated subtilisin has the smallest rate enhancement for nucleoside acylation (other than dG) possibly due to the limited solubility of dG in THF. Recall that the thymidine-activated subtilisin is not active in apolar organic solvents (e.g., hexane); therefore, the dG−subtilisin enzyme preparation will be inactive in solvents that are unable to dissociate dG effectively from the imprinted enzyme preparation.

The fidelity of subtilisin's selectivity suggests that the nucleoside base is responsible for the selectivity change (since all the nucleosides share a common 2′-deoxyribose sugar). Furthermore, although dC has the smallest base (volume 80 Å^3) of the nucleosides employed, the dC-activated subtilisin Carlsberg is the second most active enzyme preparation for the acylation of other nucleosides (Table 3). It appears that the imprinting phenomenon does not simply involve the creation

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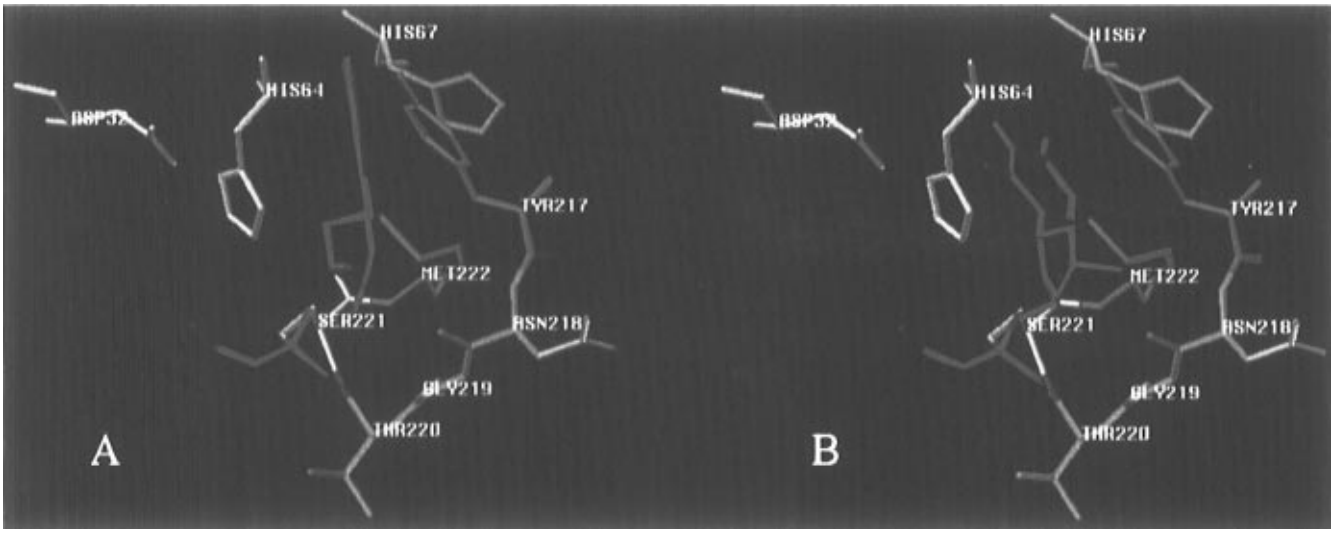

Figure 4. Molecular modeling-predicted structure of the tetrahedral intermediate for the synthesis of 5′- (A) and 3′- (B) thymidine butyrate. Both structures are the respective low-energy conformations of each systematic search. The thymidine moiety is located within the S_1' pocket in both models. The colors of the residues are the following: catalytic triad (Asp_{32}, His_{64}, and Ser_{221}) in yellow, thymidine in blue, Tyr_{217} and Met_{222} in red, and other S_1' pocket residues in green.

of a larger S_1' pocket; otherwise imprinting with larger substrates (e.g., purines) should be more effective than with smaller substrates (e.g., pyrimidines) for purine acylation (e.g., dA-activated subtilisin-catalyzed acylation of dG).

Molecular Modeling of Imprinted Subtilisin. Previous studies of imprinting have yielded to suggestions that the mechanism of imprinting-induced rate enhancement involves the conservation/alteration of the enzyme active site.[13a] Similarly, alteration of the substrate specificity via imprinting must also involve the alteration of the enzyme structure, particularly in the vicinity of the transition state. Our kinetic observations of altered substrate selectivity show that there must be a structural component to imprinting enzymes with nucleophilic substrates, and this is consistent with preliminary studies involving FTIR to probe the *secondary* structure of lyophilized proteins upon imprinting.[32]

A molecular model of the tetrahedral intermediate of thymidine acylation catalyzed by subtilisin BPN′ [33] was developed in an effort to better describe the structural aspects of the reaction. BPN′ was chosen over Carlsberg due to the greater resolution of the crystal structure in the former. Moreover, the imprinting effects on activation and selectivity obtained with BPN′ are similar to those obtained with Carlsberg. Comparison of the 5′-intermediate (Figure 4A) and 3′-intermediate (Figure 4B) shows the orientation of thymidine to be entirely within the S_1' pocket. Conformational searching of the tetrahedral intermediate (see the Materials and Methods) revealed little structural difference between acylation at the 5′- and 3′-positions for thymidine in the protein's S_1' binding pocket; the pyrimidine ring is shifted slightly within the S_1' pocket. Nearest neighbor analysis (i.e., determination of the number of protein−thymidine contacts within 2.5 Å) shows nine protein−thymidine contacts for both the 5′- and 3′-butyrates. This is consistent with the lack of observed regioselectivity in subtilisin-catalyzed thymidine acylation.

The structural similarities between the intermediates of the two regioisomers may help to explain the small degree of regioselectivity (5′-butyrate/3′-butyrate = 2.11) observed for subtilisin BPN′-catalyzed thymidine acylation. To verify our model of the tetrahedral intermediates, we compared the ratio of available conformations (this describes the steric effects on regioselectivity) for both regioisomers (see the Materials and Methods). The predicted regioselectivity of wild-type subtilisin BPN′-catalyzed thymidine acylation was 1.80, close to the observed value. Such model verification was not unique to thymidine. The experimental and predicted regioselectivities of sucrose acylation (1′-butyrate/6-butyrate) were 6.20 and 5.32, respectively. Therefore, the model utilized in this study is in close agreement with experimental results and may be useful and accurate for this study.

We again turned to our model of the enzyme covalently complexed with a nucleophilic substrate to better understand the possible structural changes introduced by imprinting. In particular, molecular dynamics provides the ability to predict the interaction of complex molecules[34] in organic solvents, where traditional analytical techniques (e.g., crystallography or spectroscopy (FTIR, CD)) are limited by the solubility of enzymes in solvents or the sensitivity of measurement. Using the low-energy conformation of thymidine 5′-butyrate (Figure 4A) as the starting structure, we employed molecular dynamics as a predictive tool to measure the interaction of thymidine with subtilisin BPN′. After equilibration for 1 ps at 0 K, the tetrahedral intermediate was heated to 100 K (in 100 fs) followed by holding at this temperature for 10 ps. The resulting model (Figure 5A) was compared to the native subtilisin structure after an identical simulation. The same methodology was used on the model of sucrose 1′-butyrate (Figure 5B), and this made it possible to distinguish between the structural changes induced by imprinting with thymidine and sucrose, respectively. Specifically, comparison of the Asp_{32} and His_{64} shows a shift of these catalytic residues in relation to the bound tetrahedral intermediate of thymidine versus sucrose. We also see a smaller change of the Asn_{155} residue's guanidino group which occupies the oxyanion hole, as well as shifts of the S_1''s Tyr_{217} and Met_{222} residues in comparing the thymidine and sucrose imprinted enzymes.

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(33) Subtilisin BPN′ was chosen for molecular modeling studies to provide a direct comparison to our previously described model for sucrose acylation.[6b] The choice of BPN′, instead of Carlsberg, also allowed for the generation of models of the site-specific mutations of the S_1' binding pocket. Indeed, the observed and predicted regioselectivities for thymidine acylation catalyzed by various subtilisin BPN′ mutants were in close agreement. For example, the M222Q enzyme had observed and predicted 5′-butyrate/3′-butyrate ratios of 1.93 and 1.77, respectively.

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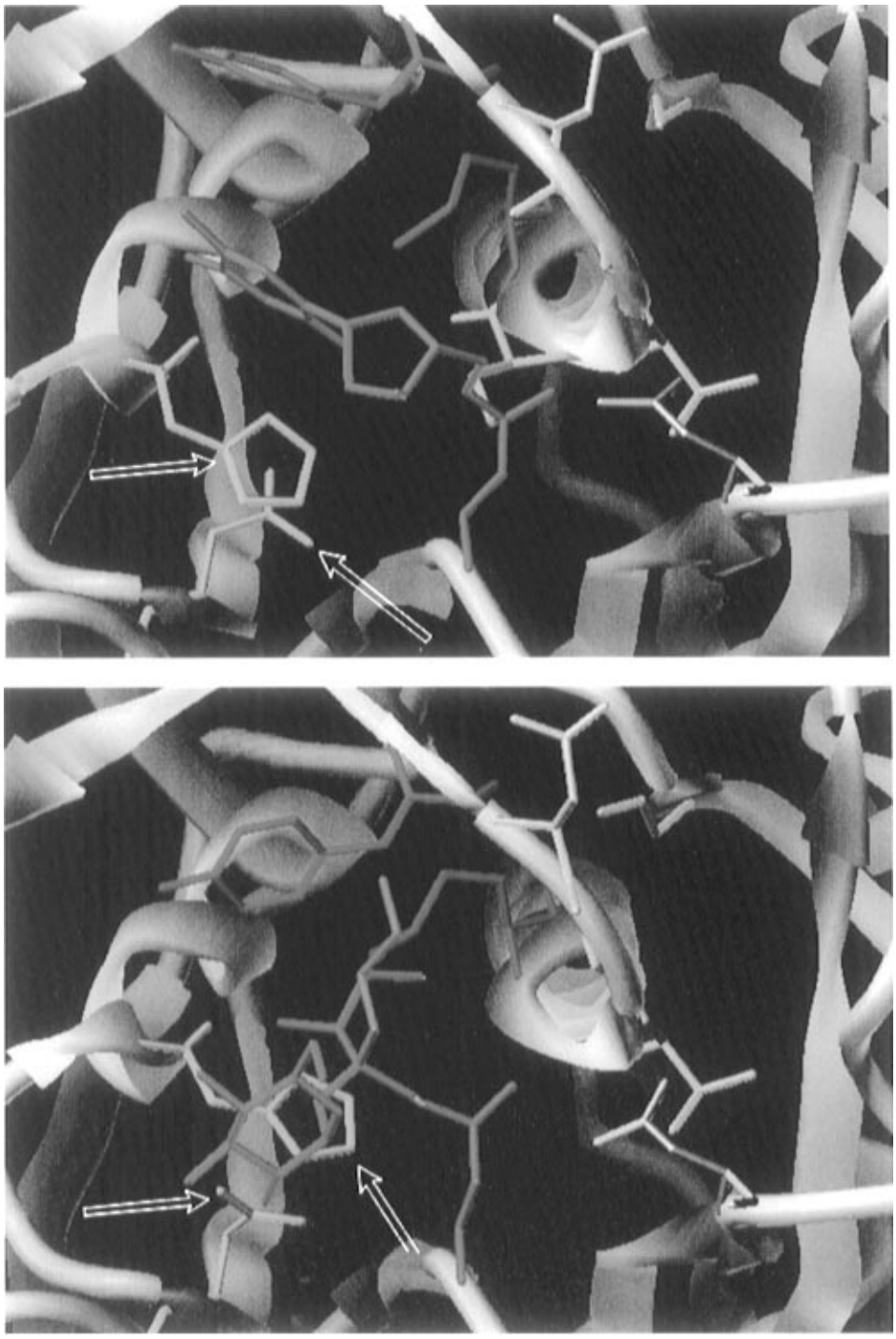

Figure 5. Molecular dynamics simulation of the effect of thymidine (A, top) and sucrose (B, bottom) on the active site structure of subtilisin BPN′. Significant changes occurred in the side chains of the catalytic residues (Asp_{32} and His_{64}) and S_1' pocket residues (Tyr_{217} and Asn_{218}). The colors of the residues are the following: catalytic triad (Asp_{32}, His_{64}, and Ser_{221}) in green, Asn_{155} in white, thymidine (A) or sucrose (B) in blue, Tyr_{217} and Met_{222} in red, and other S_1' pocket residues in yellow. The α-carbon backbone is displayed as a yellow ribbon. The arrows locate Asp_{32} and His_{64}.

To compare the respective effects of thymidine and sucrose on the active-site structure of subtilisin BPN′, we eliminated the structural changes in native subtilisin by evaluating the difference root mean square (DRMS) of the root mean square (RMS) of each model (e.g., $RMS_{(\text{thymidine imprint}-\text{native})} - RMS_{(\text{sucrose imprint}-\text{native})}$). Significant changes (Figure 6) in the structure of the enzyme (this crystal structure of subtilisin BPN′ was resolved at 2.2 Å,[35] which corresponds to the atoms being placed at ca. ±0.4 Å[36]) were observed in the residues of the catalytic triad (Asp_{32} and His_{64}) and the S_1' pocket (Tyr_{217} and Asn_{218}). Structural changes in the catalytic residues induced by substrate binding are consistent with previous modeling of substrate interactions with serine proteases (e.g., chymotrypsin and subtilisin).[37] Interestingly, the large change of the Tyr_{217} residue's position as a result of imprinting with thymidine or

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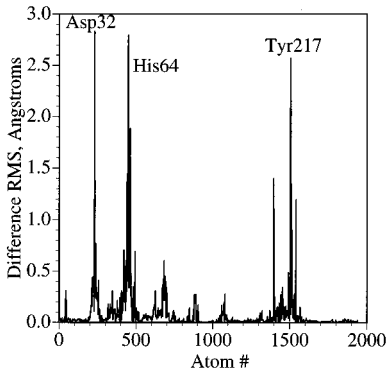

Figure 6. Difference root mean square deviation (DRMS) of the thymidine- and sucrose-imprinted enzymes. The DRMS is the difference RMS of the two models and the native subtilisin model ($RMS_{(thymidine\ imprint-native)} - RMS_{(sucrose\ imprint-native)}$). Significant changes are only those with DRMS > ca. 1 Å, since the starting structure was solved at 2.2 Å resolution.

sucrose (Figure 5) indicates that the imprinter does have a structural effect on at least one residue in the S_1' binding pocket. Such an effect, however, does not apparently impact the rate enhancement due to imprinting. As discussed previously, the Y217L mutant has the same rate enhancement for thymidine acylation as the wild-type enzyme.

The predicted structural differences in Asp_{32} and His_{64} for the thymidine- and sucrose-imprinted subtilisins suggest an explanation of the observed increase of k_{cat} (with no significant changes in K_m) by freeze-drying subtilisin with thymidine (Table 1). The model developed by Genest and Ptak[37] showed that active-site structural perturbations brought about by substrate binding to subtilisin Novo were necessary to allow for proton transfer involved in serine protease catalysis. Indeed, the most significant changes we observed in our molecular dynamics simulation involved two of the catalytic residues; the third residue of the catalytic triad, Ser_{221}, was spatially fixed in our analysis. It appears, then, that the primary mechanism of imprinting on the improvement in catalytic performance involves the alteration of the catalytic triad structure, as opposed to catalytically significant rearrangement of the S_1' binding pocket.

Conclusions

The enhacement of catalytic performance of enzymes in organic solvents provides additional opportunities to expand their use in commercial synthetic organic chemistry. We have shown that lyophilizing subtilisin in the presence of nucleophilic substrates increases the catalytic rate for both nucleoside and carbohydrate acylation reactions in organic media, including the first known report of imprinting enzymes by lyophilizing subtilisin in the presence of nucleosides. The activation of enzymes by imprinting appears to be the result of structural changes in the catalytic triad (as predicted by molecular dynamics simulations), and this further results in an observed increase in k_{cat}. The rate enhancement due to imprinting with nucleophilic compounds appears to be a general phenomenon, the only apparent limitation to this being that the imprinter must be soluble in aqueous solution and must also be soluble in the organic solvent reaction medium to remove the imprint molecule from the enzyme. The imprinted enzyme is stable to storage; at −20 °C, both the sucrose- and thymidine-imprinted subtilisins Carlsberg and BPN′ retained their imprinting-induced activations and selectivities for greater than 2 months.

In addition to an increased catalytic rate, imprinting subtilisin with nucleophilic substrates allows for the rational control of enzymic substrate selectivity. In this manner, imprinting directly affects the transition state of the enzymatic reaction. Such "transition state engineering" in organic solvents complements protein engineering,[11b,18,38] catalyst engineering,[13b,39] and solvent engineering,[18a,40] and once again demonstrates that useful changes in the catalytic properties of enzymes in organic solvents need not involve tedious procedures.

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